AN INVESTIGATION OF THE ACUTE AND LONGER-TERM EFFECTS OF 3,4-METHYLENEDIOXYMETHAMPHETAMINE (MDMA; 'ECSTASY').

Annis Olivia Mechan

A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy
in the Faculty of Applied Sciences
of De Montfort University.

November 2001

Department of Pharmacology
School of Pharmacy
De Montfort University
Leicester

INDEX

TABLES AND FIGURES

ABSTRACT

Administration of (±)3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') to rats produces an acute release of cerebral 5-hydroxytryptamine (5-HT) and dopamine, an acute hyperthermic response, and long-term selective neurotoxic depletion of regional brain 5-HT. In the studies reported in this thesis, administration of a single dose of MDMA (12.5 mg/kg i.p.) to Dark Agouti rats produced a 20 - 40 % depletion of regional brain 5-HT and 5-HIAA content one week later. The functional consequences of this loss were investigated by assessing the effects on anxiety-related behaviour and thermoregulation. The acute hyperthermic response was investigated by administering compounds affecting serotonergic or dopaminergic function. In contrast to rats, MDMA induces a selective long-term dopaminergic depletion in mouse brain. The mechanisms involved in this depletion were investigated using putative neuroprotective compounds.

MDMA pretreatment markedly reduced "anxiety", 80 days post-treatment, as measured in the elevated plus-maze and open-field. Changes did not become apparent until over two months after MDMA administration, indicating that the changes are not solely due to the cerebral 5-HT loss (evident one week post-treatment), but that adaptive changes also occur.

When rats given a single dose of MDMA (12.5 mg/kg.) 5-6 weeks earlier were exposed to high ambient temperature (30 ± 0.5 °C) for 1 h, they displayed a faster rise in rectal temperature, and sustained hyperthermia when returned to a normal temperature environment (20 ± 2 °C). These data demonstrate that a long-term defect in thermoregulation is produced by a modest MDMA-induced loss of cerebral 5-HT.

Investigation of the acute MDMA-induced hyperthermia revealed that 5-HT antagonists and uptake inhibitors had little effect on the hyperthermia, even though fluoxetine almost completely abolished MDMA-induced release of hippocampal 5-HT. The dopamine D_1 antagonist, SCH 23390, dose-dependently attenuated the hyperthermia, indicating that 5-HT is probably not directly involved in the response, but that dopamine plays a role via an action at D_1 receptors.

The selective neuronal nitric oxide synthase (nNOS) inhibitors, AR-R17477AR and S-methylthiocitrulline, protected against MDMA-induced dopaminergic depletion in mice, indicating the involvement of nNOS in the neurotoxic pathway. The free radical scavenger, α-phenyl-N-tert-butyl nitrone, was not protective, although there is evidence of MDMA-induced free radical generation. Since neither of the N-methyl-D-aspartate antagonists, AR-R15896AR and MK-801, were neuroprotective, free radicals are probably not being formed as a result of increased glutamatergic activity at NMDA receptors, but rather auto-oxidation of MDMA metabolites.

In conclusion, the studies demonstrated that administration of a single dose of MDMA, producing a 20 - 40 % regional brain depletion of 5-HT in rats, induced long-term functional deficits. These findings have important clinical implications, since a similar loss of cerebral 5-HT has also been reported to occur in the brains of human recreational users of MDMA.

ACKNOWLEDGEMENTS

I wish to thank Professors A. Richard Green and J. Martin Elliott for giving me the opportunity to study for a Ph.D. They have both provided endless enthusiasm, support and encouragement throughout the past three years and I am eternally grateful to both of them. I am also grateful for the numerous opportunities to present my data at national and international conferences and wish to thank the Serotonin Club, which provided a travel bursary to enable me to attend the Serotonin Club and Society for Neuroscience meetings in New Orleans (November, 2000), and the British Pharmacological Society, which provided a travel bursary to enable me to attend the BPS meeting in Dublin (July 2001).

I wish to thank several members of staff in the School of Psychology, University of Leicester, for their advice and support. In particular, I wish to thank Dr Paula Moran, who taught me the finer arts of behavioural testing paradigms and statistical analysis and both Professor Michael Joseph and Dr Andrew Young, who brought me to Leicester in the first place.

For technical advice and assistance, I wish to thank Martin Edwards, Anita O'Donoghue and Steve Liquorish in the Department of Pharmacology and Evelyn Topping at the Leicester Royal Infirmary.

I wish to thank Professor Paul Whiting for permitting me to work under his Project Licence.

I wish to thank Dr Esther O'Shea, University of Nottingham, for help with dissections and h.p.l.c., Dr Tyra Zetterström, who first taught me how to do h.p.l.c., and Nicola Wilsher, without whom the 'h.p.l.c. monster' would not still be running.

I wish to thank Steve Bennett and Tom Beveridge for their 'expert' assistance and patience.

I wish to thank Dr Blanca Esteban, Universidad Complutense, Madrid who, during the three months she spent at De Montfort University, provided extensive help and encouragement, someone to talk to during long hours of experimentation, and taught me to be more organised. I also wish to thank Dr Maribel Colado, for allowing me to report some of her data, for performing statistical analysis on some of my data and for teaching her students, Esther and Blanca, so well.

I wish to thank my sister, Charlotte, and my mother-in-law, Joyce, for their patience and hard work as proof-readers.

I wish to thank all my friends for their support and encouragement.

I wish to thank my siblings – Charlotte and Jonathan – who have put up with me being a student for eternity.

I wish to thank my parents – Derek (the first Dr Mechan) and Ruth – who have supported me emotionally (and financially) throughout my educational pursuits and have enabled me to achieve my dreams.

Finally, I wish to thank Andrew, my husband, for being him.

PUBLICATIONS

Publications resulting from work described in this thesis:

PAPERS

MECHAN, A.O.; O'SHEA, E.; ELLIOTT, J.M.; COLADO, M.I.; GREEN, A.R. (2001). A neurotoxic dose of 3,4-methylenedioxymethamphetamine (MDMA; ecstasy) to rats results in a long term defect in thermoregulation. *Psychopharmacology*, **155**, 413-418.

MECHAN, A.O.; MORAN, P.M.; ELLIOTT, J.M.; YOUNG, A.M.J.; JOSEPH, M.H.; GREEN, A.R. A study of the effects of a neurotoxic dose of 3,4-methylenedioxymethamphetamine (MDMA; ecstasy) on the long-term behaviour of rats in the plus maze and open field. *Psychopharmacology* (in press).

MECHAN, A.O.; MORAN, P.M.; ELLIOTT, J.M.; YOUNG, A.M.J.; JOSEPH, M.H.; GREEN, A.R. Dark Agouti rats show a marked difference to Sprague-Dawley rats in their behaviour on the elevated plus-maze, open-field apparatus and automated activity meters, and in their response to diazepam. *Psychopharmacology* (in press).

COLADO, M.I.; CAMARERO, J; MECHAN, A.O.; SANCHEZ, V; ESTEBAN, B; ELLIOTT, J.M.; GREEN, A.R. A study of the mechanisms involved in the neurotoxic action of 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') on dopamine neurones in mouse brain. *British Journal of Pharmacology* (in press).

MECHAN, A.O.; COLADO, M.I.; ESTEBAN, B.; ELLIOTT, J.M.; GREEN, A.R. The pharmacology of the acute hyperthermic response that follows administration of 3,4-methylenedioxymethamphetamine to rats. *British Journal of Pharmacology* (in press)

ABSTRACTS

MECHAN, A.O.; ESTEBAN, B; COLADO, M.I.; ELLIOTT, J.M.; GREEN, A.R. (2001). Studies on the mechanisms involved in the degeneration of dopamine nerve endings in mouse brain following administration of MDMA ('Ecstasy'). *British Journal of Pharmacology*, **134**, 142P

MECHAN, A.O.; GREEN, A.R.; ELLIOTT, J.M.; YOUNG, A.M.J.; JOSEPH, M.H.; MORAN, P.M. (2001). A study of the differences in the behaviour of Dark Agouti and Sprague Dawley rats in the elevated plus-maze. *British Journal of Pharmacology*, **133**, 184P.

MECHAN, A.O.; COLADO, M.I.; ESTEBAN, B.; ELLIOTT, J.M.; GREEN, A.R. (2000). Evidence against MDMA-induced hyperthermia being mediated by 5-HT release in the brain. *British Journal of Pharmacology*, **131**, 153P

MECHAN, A.O.; ELLIOTT, J.M.; GREEN, A.R.; YOUNG, A.M.J.; JOSEPH, M.H.; MORAN, P.M. (2000). The long-term effects of (±) 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') on "anxiety" in the Dark Agouti rat. *Serotonin Club/Brain Research Bulletin conference, 'Serotonin: From the molecule to the clinic'*, P177

MECHAN, A.O.; COLADO, M.I.; ESTEBAN, B.; ELLIOTT, J.M.; GREEN, A.R. (2000). The pharmacology of MDMA ('Ecstasy')-induced hyperthermia in the Dark Agouti rat. *Serotonin Club/Brain Research Bulletin conference, 'Serotonin: From the molecule to the clinic'*, P178.

MECHAN, A.O.; GREEN, A.R.; ELLIOTT, J.M.; YOUNG, A.M.J.; JOSEPH, M.H.; MORAN, P.M. (2000). Long-term effects of MDMA ('Ecstasy') on anxiety in the Dark Agouti rat in the plus-maze and open-field. *Society for Neuroscience 30th Annual Meeting*, 721.15

MECHAN, A.O.; ELLIOTT, J.M.; GREEN, A.R.; YOUNG, A.M.J.; JOSEPH, M.H.; MORAN, P.M. (2000). An investigation of the long-term effects of (±) 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') on anxiety in the Dark Agouti rat in the plus-maze and open field. *European Journal of Neuroscience*, **12**, Suppl. 11, 216.12

MECHAN, A.O.; ELLIOTT, J.M.; COLADO, M.I.; GREEN, A.R. (1999). Altered thermoregulatory response to high ambient temperature (30 °C) in MDMA-pretreated rats. *British Journal of Pharmacology*, **128**, 185P.

FULL PAPERS IN PREPARATION

BEVERIDGE, T.J.R.; MECHAN, A.O.; SPRAKES, M.; PEI, Q.; ZETTERSTRÖM, T.S.C.; GREEN, A.R. & ELLIOTT, J.M. Effect of a prior neurotoxic dose of MDMA on the expression of the functional immediate-early gene arc induced by subsequent acute MDMA administration.

ABBREVIATIONS

%	percentage
ΔpH	transmembrane pH difference
ΔT	change in temperature
-/-	homozygous knockout mice
[^{123}I]β-CIT	2β-carbomethoxy-3β-(4-iodophenyl)tropane
[^{125}I]MIL	*N* 1-methyl-2-[^{125}I]lysergic acid diethylamide
[^{3}H]	tritiated
"	inch(es)
° C	degrees Centigrade
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
µM	micromolar
^{1}H MRS	proton magnetic resonance spectroscopy
3-NT	3-nitrotyrosine
5,7-DHT	5,7-dihydroxytryptamine
5-CT	5-carboxamidotryptamine
5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	5-hydroxytryptamine; serotonin
5-HTP	5-hydroxy-L-tryptophan
5-MeODMT	5-methoxy-*N,N*-dimethyltryptamine
6-HO-MDA	2-hydroxy-4,5-(methylenedioxy)amphetamine
6-HO-MDMA	2-hydroxy-4,5-(methylenedioxy)methamphetamine
6-OH-DA	6-hydroxydopamine
7-NI	7-nitroindazole
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)tetralin
Å	angstroms
ACh	acetylcholine
amp	amplitude
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPT	α-methyl-p-tyrosine
ANOVA	analysis of variance
AR-R15896AR	S-(+)-α-phenyl-2-pyridine ethanamide dihydrochloride
AR-R17477AR	N-(4-(2-((3-chlorophenylmethyl) amino)-ethyl)phenyl) 2-thiophene carboxamidine
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene

BRL 43694	endo-N-[9 methyl-9-azabicylo (3,3,1)non-3-yl]-1-methyl-indazole-3 carboxamide
BW 723C86	1-[5-(2-thienylmethoxy)-1H-3-indoyl] propan-2-amine hydrochloride
Ca^{2+}	calcium ions
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CCD	charged coupled device
cGMP	cyclic guanosine monophosphate
CGS 19755	*cis*-4-(phosphonomethyl)-2-piperidine carboxylic acid
CHO	choline compounds
Cl^-	chloride ion(s)
cm	centimetre(s)
cm^2	square centimetre(s)
cm^3	cubic centimetre(s)
CMZ	Clomethiazole edisilate; (Chloro-2-ethyl)-5-methyl-4 thiazole ethanedisulfonate-1,2
CNS	central nervous system
COMT	catechol-O-methyltransferase
COS	transformed monkey kidney fibroblast cells
CP 93,129	3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one
crf	continuous reinforcement
CS	conditioned stimulus
CSF	cerebrospinal fluid
Cu	copper
DA	Dark Agouti rats
DDC	diethyldithiocarbamate
DEA	Drug Enforcement Administration
DHA	3,4-dihydroxyamphetamine; α-methyldopamine
DHBA	dihydroxybenzoic acid
DHMA	3,4-dihydroxymethamphetamine; *N*-methyl-α-methyldopamine
DIC	disseminated intravascular coagulation
DMSO	dimethylsulphoxide
DOI	(±)-2,5-dimethoxy-4-iodoamphetamine
DOPAC	3,4-dihydroxyphenylacetic acid
dopamine	3-hydroxytyramine hydrochloride
DSP_4	*N*-(2-chloroethyl)-*N*-ethyl-2-bromo benzylamine
EDTA	diaminoethanetetra acetic acid
EWL	evaporative water loss
$FeCl_2$	ferrous chloride

FeTMPyP	5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron III
FG7142	N-methyl-β-carboline-3-carboxamide
FR	fixed ratio
g	gram(s)
GABA	γ-aminobutyric acid
GBL	γ-butyrolactone
GBR 12909	1-[2-*bis* (4-fluorophenyl) methoxy]ethyl]-4-3-phenylpropyl]piperazine
GFAP	glial fibrillary acidic protein
GI tract	gastrointestinal tract
GLX	glutamate/glutamine
GR 113808	1-[2-(methylsulphonylamino)ethyl]4-piperidinyl]methyl-1-methyl-indole-3 carboxylate
GR 127935	2'-methyl-4'-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide
GR38032F	1,2,3,9-tetra-hydro-q-methyl-3-[(2-methyl-1H-imadizol-l-yl) methyl]d-4H-car-bazol-4-one hydrochloride•$2H_2O$
h	hour(s)
h.p.l.c.	high performance liquid chromatography
H^+	hydrogen ion(s)
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloride
HEK 293	human embryonic kidney 293 cells
HeLa	human epithelial cells (cervical cancer)
HL	Hooded Lister rats
HMA	4-hydroxy-3-methoxyamphetamine
HMMA	4-hydroxy-3-methoxymethamphetamine
HVA	4-hydroxy-3-methoxy-phenylacetic acid; homovanillic acid
Hz	Hertz
I	iodine
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
ICS-205-930	[3α-tropanyl]-1H-indole-3-carboxylic acid ester
Ins*P*	inositol triphosphate
K^+	potassium ion(s)
KCl	potassium chloride
kg	kilograms
KH_2PO_4	potassium dihydrogen orthophosphate

L 694247	2-[5-[3-(4-methylsulphonylamino)benzyl-1 2,4-oxadiazol-5-yl]-1H-indol-3-yl] ethanamine
L-DOPA	3,4-dihydroxyphenylalanine
LEW	Lewis rats
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMA	L-nitro-methylarginine
L-NOARG	N^{ω}-nitro-L-arginine
LSD	lysergic acid diethylamide
m	metre(s)
M	molar
mA	milliamp(s)
MAO	monoamine oxidase
m-CPP	1-(3-chlorophenyl)piperazine
MDA	3,4-methylenedioxyamphetamine
MDBA	3,4-methylenedioxybutylamphetamine
MDEA	N-ethyl-3,4-methylenedioxyamphetamine; 'Eve'
MDL 100,907	R-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol
MDL 11,939	α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol
MDL72222	[1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate methyl sulphate hemihydrate
MDMA	3,4-methylenedioxymethamphetamine; 'Ecstasy'
mescaline	3,4,5-trimethoxyphenethylamine
mg	milligram(s)
$MgCl_2$	magnesium chloride
MI	*myo*-inositol
min	minute(s)
MK-801	(5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; dizocilpine
ml	millilitre(s)
mM	millimolar
mPFC	medial prefrontal cortex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR	metabolic rate
MRI	magnetic resonance imaging
ms	millisecond(s)
mV	millivolts(s)
nA	nanoamps(s)
NA	N-acetylaspartate
Na^+	sodium ion(s)

NaCl	sodium chloride
N-Arg	L-N^G-nitro-arginine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
ng	nanograms(s)
NIH/S	NIH "Swiss"-type Albino mice
NMDA	N-methyl-D-aspartate
N-Me-α-MeDA	*N*-methyl-α-methyldopamine
nMol	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
$NO^{\bullet}$	nitric oxide free radical
NO^{+}	nitric oxide free radical
NOS	nitric oxide synthase
O_2	oxygen
O_2^{-}	superoxide anion
$OH^{\bullet}$	hydroxyl free radical
$ONOO^{-}$	peroxynitrite radical
OSA	octanesulphonic acid disodium salt
p.o.	per oral
PBN	α-phenyl-N-tert-butyl nitrone
PCA	*para*-chloroamphetamine
PCPA	*para*-chlorophenylalanine
PEG	polyethylene glycol
PET	positron emission tomography
PKC	protein kinase C
r.p.m.	revolutions per minute
Ro 60 0175	(*S*)-2-chloro-5-fluoro-indol-1-yl)-1-methyl ethylamine fumarate
s	second(s)
s.c.	subcutaneous
s.e.m.	standard error of the mean
SAP	stretched attend posture
SB 200646	N-(1-methyl-5-indolyl)-N'-(3-pyridyl) urea
SB 204070	8-amino-7-chloro-(N-butyl-4-piperidyl)methylbenzo-1,4-dioxan-5-carboxylate
SB 206553	5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrol[2,3-f]indole
SB 242084	6-chloro-5-methyl-methyl-1-[2-(-methylpyridyl-3-oxy)-pyrid-5-yl carbamoyl]indoline
SCH 23390	R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzapine

SD	Sprague Dawley rats
SDZ 21009	4(3-terbutylamino-2-hydroxypropoxy)indol-2-carbonic acid-isopropylester
SERT	serotonin transporter
SHR	Spontaneously hypertensive rats
S-MTC	S-methylthiocitrulline
SOD	superoxide dismutase
SPECT	single photon emission computed tomography
t	time
T_a	ambient temperature
TBARS	thiobarbituric acid-reacting substance
TCP	tranylcypromine
TH	tyrosine hydroxylase
THC	Δ9-tetrahydrocannabinol
TPH	tryptophan hydroxylase
Tri-HO-A	2,4,5-trihydroxyamphetamine
Tri-HO-MA	2,4,5-trihydroxymethamphetamine
TV	television
U.K.	United Kingdom
U.S.	United States
UCS	unconditioned stimulus
V	volts
VI	variable interval
vs.	versus
W	watts
w/v	weight to volume ratio
WAY 100,635	*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide
WAY 100135	N-tert-butyl-3-[4-(2-methoxyphenyl) piperazin-1-yl]-2-phenylpropanamide
Zn	zinc
α	alpha
β	beta
γ	gamma
Δ	delta

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 ORIGINS AND RECREATIONAL USE OF MDMA

1.1.1 History of MDMA

3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') is a ring-substituted amphetamine derivative (see Figure 1.1), which is structurally related to both amphetamine and the hallucinogenic compound, 3,4,5-trimethoxyphenethylamine (mescaline). MDMA has often been reported to have been originally synthesised for use as an appetite suppressant (see Christophersen, 2000; Dowling *et al.*, 1987; Green *et al.*, 1995; Henry, 1992; Henry *et al.*, 1992; Rattray, 1991; Steele *et al.*, 1994). However, according to Cohen (1998), "MDMA was actually patented in Germany in the 1900s as a precursor agent - or intermediate structural compound - possessing properties deemed to contain primary constituents for therapeutically active compounds", and was never intended for use as an anorectic drug. In the 1970s, MDMA was reported to have been used in psychotherapy, where it was seen to increase patient self-esteem and facilitate therapeutic communication. In such settings, MDMA was orally administered at a dose of 75 - 175 mg and was reported to have some acute sympathomimetic side effects, such as increased heart rate and blood pressure, and transient anxiety (see Greer & Strassman, 1985; Grinspoon & Bakalar, 1986).

Whatever its origins MDMA has, in more recent years, gained popularity as a recreational drug (see Christophersen, 2000; Green *et al.*, 1995; Hegadoren *et al.*, 1999; Morgan, 2000; Parrott & Lasky, 1998; Peroutka, 1997; Randall, 1992; Rattray, 1991; Solowij *et al.*, 1992; Steele *et al.*, 1994). In 1985, the U.S. Drug Enforcement Administration (DEA) classified MDMA as a Schedule 1 drug due to its high abuse potential, lack of clinical application, lack of accepted safety for use under medical supervision (see www.usdoj.gov/dea), and evidence that the related compound, 3,4-methylenedioxyamphetamine (MDA), induced serotonergic nerve terminal degeneration in rat brain (Ricaurte *et al.*, 1985). In the U.K., possession of MDMA is also illegal, being controlled as a Class A drug under the Misuse of Drugs Act (1971) (see www.drugscope.org.uk).

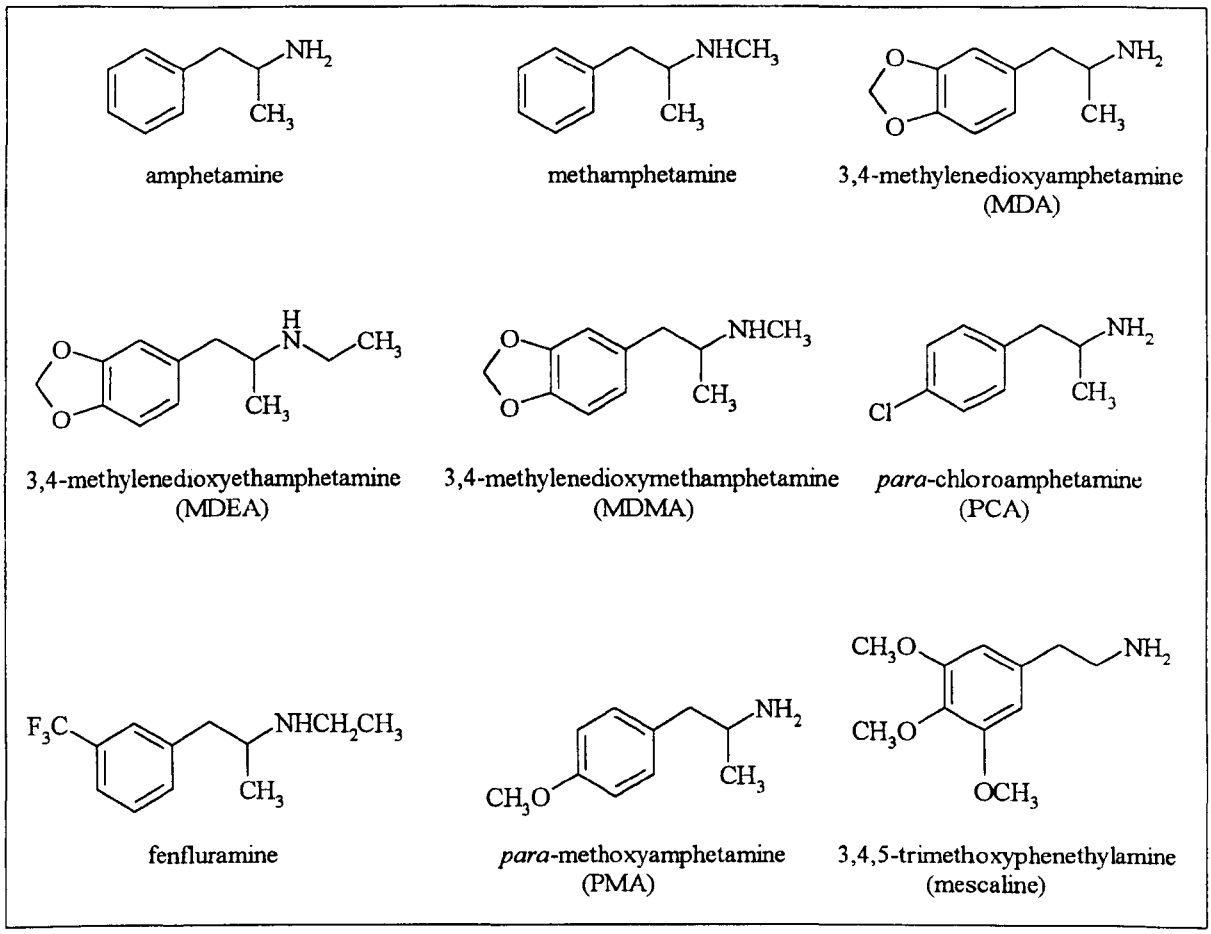

Figure 1.1 Chemical structures of amphetamine, some of its derivatives and the hallucinogen, mescaline.

1.1.2 Recreational use of MDMA

MDMA first gained popularity as a recreational drug in the early 1980s, especially among college students (see Peroutka, 1987), usually being taken at "rave" or "techno" parties, particularly in large dance clubs, although there is a reported increase of use in smaller party environments (www.usdoj.gov/dea). MDMA usually produces a relaxed, euphoric state, including emotional openness, empathy, reduction of negative thoughts and a decrease in inhibitions (see Davison & Parrott, 1997; Green *et al.*, 1995; Hegadoren *et al.*, 1999; Henry, 1992; Liechti & Vollenweider, 2000; Morgan, 2000; Parrott & Stuart, 1997; Peroutka *et al.*, 1988; dancesafe.org; www.drugscope.org.uk; www.erowid.org; www.thesite.org; www.usdoj.gov/dea). One of the major acute physiological effects of MDMA use is an increase in body temperature, which is potentially extremely dangerous when taking the drug in a hot, crowded environment, and can result in heatstroke. Therefore, users are recommended to take regular breaks from dancing and to sip approximately one pint of non-alcoholic fluid over the course of each hour. "Rave" venues often have "chill out" rooms, which enable individuals to take breaks from dancing in a less crowded, relaxed atmosphere (see Henry, 1992; www.dancesafe.org; www.drugscope.org.uk; www.thesite.org).

1.2 EFFECTS OF MDMA IN EXPERIMENTAL ANIMALS

1.2.1 Acute effects

The acute effects which follow MDMA administration to rats include: (1) a hyperthermic response, (2) a release of 5-hydroxytryptamine (5-HT; serotonin), (3) alterations in 5-HT synthesis, (4) a release of dopamine, (5) free radical production (6) neuroendocrine changes, (7) cardiovascular responses, and (8) behavioural responses. Acute 5-HT and dopamine release and behavioural responses have also been demonstrated in mice.

1.2.1.1 Acute hyperthermia and thermoregulatory responses

(1) Hyperthermia under 'normal' ambient temperature conditions

Under 'normal' ambient temperature (T_a) conditions (20 - 22 °C), MDMA administration to rats tends to result in a marked hyperthermic response of approximately +1 - 2 °C, which peaks at about 40 - 60 min post-injection (see Broening *et al.*, 1995; Dafters, 1994; 1995; Malberg *et al.*, 1996; O'Shea *et al.*, 1998; Shankaran & Gudelsky, 1999). Similar responses are observed following administration of MDMA (see Johnson *et al.*, 2000; Miller & O'Callaghan, 1994; 1995; O'Callaghan & Miller, 1994), methamphetamine (see Albers & Sonsalla, 1995; Ali & Itzhak, 1998; O'Callaghan & Miller, 1994), or MDA (see Miller & O'Callaghan, 1994; O'Callaghan & Miller, 1994) to mice, in addition to administration of methamphetamine (see Green *et al.*, 1992; Metzger *et al.*, 2000), or MDEA (see Colado *et al.*, 1999d) to rats.

While the majority of studies demonstrate an acute hyperthermic response to MDMA administration under 'normal' T_a conditions, an acute decrease in temperature has also been reported. For example, Malberg & Seiden (1998) administered MDMA (20 or 40 mg/kg s.c.) to male Holtzman rats and demonstrated a hypothermic response at a T_a of 20 - 22 °C, no change from control animals at a T_a of 24 - 26 °C, and a hyperthermic response at a T_a of 28 - 30 °C. Thus relatively small changes in T_a were demonstrated to produce marked changes in the temperature response to MDMA administration (Malberg & Seiden, 1998). O'Shea *et al.* (2001) administered MDMA (10, 20 or 30 mg/kg i.p.) to male Swiss-Webster mice, three times at 3 h intervals, and observed a hypothermic response following the first injection at doses of 10 and 20 mg/kg, while hyperthermia was observed following the first injection at a dose of 30 mg/kg, and following the second injection at a dose of 20 mg/kg.

(2) Involvement of ambient temperature in the hyperthermic response

A number of authors have further demonstrated the importance of ambient temperature in the changes in body temperature (Broening *et al.*, 1995; Dafters, 1994; 1995; Dafters & Lynch, 1998; Farfel & Seiden, 1995a; Gordon *et al.*, 1991), and thermoregulatory responses (Gordon *et al.*, 1991) induced by MDMA. For example, Dafters (1994)

administered MDMA (2.5, 5 or 7.5 mg/kg s.c.) to male Wistar rats and measured temperature for the following 10 h. The first experiment comprised animals being housed under T_a conditions of either 11 or 24 °C for a total of 48 h, commencing 24 h before drug administration. At a T_a of 11 °C, a dose-dependent hypothermic response was observed; a maximal decrease in temperature of approximately -1 °C was seen in animals administered 7.5 mg/kg MDMA. At a T_a of 24 °C, a dose-dependent hyperthermic response was seen; administration of 7.5 mg/kg MDMA resulted in an increase in temperature of approximately +1.75 °C. During the second experiment, rats were administered MDMA (2.5, 5 or 7.5 mg/kg s.c.) under T_a conditions of 24 °C, 30 min before half of each treatment group were transferred to a "cool" room (T_a = 11 °C) for 90 min. Hyperthermia was significantly attenuated in animals exposed to the cool environment; maximal hyperthermic responses were approximately half the value of those seen in animals kept at 24 °C. Thus, dose-related changes in body temperature induced by MDMA appear to be dependent on T_a (Dafters, 1994).

Dafters & Lynch (1998) investigated the effects of more subtle changes in T_a (a reduction of 5 °C below the temperature of the animal holding room, from 22 °C to 17 °C) on MDMA-induced changes in body temperature ("thermogenic response"). Animals which were administered MDMA (10 or 15 mg/kg s.c.) at a T_a of 17 °C were placed in a temperature-controlled test room for a total of 4 h (1 h prior to drug treatment and 3 h post-treatment). This resulted in a hypothermic response, where body temperature decreased by approximately -2 °C. At 22 °C, however, an increase in body temperature of approximately +1 °C was observed. The authors suggested that such a high degree of sensitivity to T_a indicated that MDMA has some effect on the thermoregulatory set-point mechanism, which they believed was compatible with the fact that MDMA administration results in an acute release of serotonin and subsequent stimulation of central serotonergic pathways, and that serotonergic pathways are involved in thermoregulation (Dafters & Lynch, 1998).

Gordon *et al.* (1991) administered MDMA (30 mg/kg s.c.) to male Long-Evans rats and investigated the effects on their thermoregulatory systems, by monitoring metabolic rate (MR), evaporative water loss (EWL) and rectal temperature, under three T_a conditions

(10, 20 and 30 °C). MR was significantly increased, compared to control animals, under T_a conditions of 20 and 30 °C and was unchanged at 10 °C. MDMA-treated rats demonstrated an increasing EWL with increasing T_a; EWL values in MDMA-treated rats were approximately 275 % above control values, at a T_a of 30 °C. Rectal temperature increased with increasing T_a: (1) hypothermia (-2 °C) occurred at 10 °C, (2) at 20 °C there was no difference between MDMA- and saline-treated animals, and (3) at 30 °C, hyperthermia was seen (+2 °C). The authors thus demonstrated that MDMA administration had profound effects on the thermoregulatory system of the rat, involving increases in MR, EWL and rectal temperature, and that such effects were apparently dependent on T_a (Gordon *et al.*, 1991).

(3) ***Pharmacological inhibition of the hyperthermic response***

Prevention of the MDMA-induced acute hyperthermic response in both rats and mice provides neuroprotection against toxicological damage (see Chapters 6 and 7). Chapter 6 of this thesis reports an investigation of the acute hyperthermic response following MDMA administration to Dark Agouti (DA) rats, which involved the administration of a series of drugs which alter either 5-HT or dopamine function. Chapter 7 reports an investigation of the mechanisms of MDMA-induced neurotoxicity in mice, and demonstrates that PBN, for example, provided neuroprotection against MDMA-induced dopamine loss only where the hyperthermic response was blocked.

1.2.1.2 Acute release and depletion of 5-HT

MDMA administration to rats has been demonstrated to induce an acute release of 5-HT both *in vivo* (Gough *et al.*, 1991; Gudelsky & Nash, 1996; Mechan *et al.*, 2001b; Nixdorf *et al.*, 2001; Sabol & Seiden, 1998; Shankaran & Gudelsky, 1999; Yamamoto *et al.*, 1995) and *in vitro* (see Berger *et al.*, 1992; Crespi *et al.*, 1997; Johnson *et al.*, 1986; Koch & Galloway, 1997; O'Loinsigh *et al.*, 2001; Schmidt, 1987a; 1987b; Schmidt *et al.*, 1987). In addition, a number of studies have been reported where 5-HT concentrations have been measured in brain tissue during the first few hours after drug administration, in both rats (see Aguirre *et al.*, 1995; Colado & Green, 1994; Gough *et al.*, 1991; Logan *et al.*, 1988; McKenna *et al.*, 1990; Schmidt, 1987a; 1987b; Schmidt &

Kehne, 1990; Schmidt *et al.*, 1986; 1987; 1990c; Stone *et al.*, 1987a; 1987b) and mice (see Logan *et al.*, 1988; O'Shea *et al.*, 2001; Stone *et al.*, 1987a).

(1) In vivo 5-HT release

Gudelsky & Nash (1996) demonstrated a dose-related increase in extracellular 5-HT concentrations in the striatum and medial prefrontal cortex, following peripheral administration of MDMA (2.5, 10 or 20 mg/kg i.p.). Striatal 5-HT release was significantly attenuated by pretreatment with the serotonin uptake inhibitor, fluoxetine, indicating that MDMA-induced 5-HT release involves a carrier-mediated mechanism. Co-administration of 5-hydroxytryptophan (5-HTP; 5-HT precursor) and carbidopa (DOPA decarboxylase inhibitor) produced a significant increase in striatal extracellular 5-HT, a response which was enhanced ten-fold by administration of MDMA. The authors suggested that these data indicated a possible action of MDMA on synaptic vesicles, since 5-HTP treatment would enhance 5-HT synthesis, while MDMA treatment led to disruption of storage mechanism and activation of the 5-HT transporter, thus leading to the pronounced increase in extracellular 5-HT (Gudelsky & Nash, 1996).

Sabol & Seiden (1998) investigated the effects of pretreatment with reserpine, which depletes vesicular dopamine stores, on MDMA-induced striatal 5-HT release. MDMA administration (10 mg/kg i.p.) resulted in an acute, massive release of 5-HT (approximately 30 times above baseline values) within 30 min. Reserpine pretreatment (10 mg/kg i.p., 18 h prior to MDMA injection) resulted in a significant attenuation of 5-HT release, however reserpine was also demonstrated to induce a marked hypothermic response. Thus the reserpine-treated animals were warmed to maintain a normal body temperature (37.9 °C), which resulted in a peak 5-HT release which was intermediate between that induced by MDMA alone and MDMA + reserpine. The authors suggested that, since maintenance of normal body temperature did not fully reinstate MDMA-induced 5-HT release, the depletion of vesicular stores rather than attenuation of hyperthermia was primarily responsible for the inhibitory effects of reserpine (Sabol & Seiden, 1998).

Shankaran & Gudelsky (1999) demonstrated an acute, significant increase in striatal extracellular 5-HT concentrations following administration of MDMA (7.5 mg/kg i.p.) to male SD rats. This response was markedly attenuated in rats which had previously been subjected to a neurotoxic dose regimen of MDMA (10 mg/kg i.p., four times at 2 h intervals). The authors suggested that this attenuation could be due to the loss of 5-HT nerve terminals induced by the neurotoxic treatment regimen, and indicated functional deficits in serotonergic neurotransmission (Shankaran & Gudelsky, 1999).

(2) In vitro 5-HT release and uptake

In vitro 5-HT release and uptake have been demonstrated using rat brain slices and synaptosomes. For example, Koch & Galloway (1997) demonstrated that incubation of MDMA (0.3 - 3 μMol) with rat striatal slices resulted in a dose-dependent increase in extracellular 5-HT, which was completely prevented by pretreatment with either fluoxetine (3 μMol) or the 5-HT_2 receptor antagonist, ketanserin (1 μMol). The inhibitory effects of fluoxetine indicated that MDMA increases extracellular 5-HT levels via a 5-HT transporter-sensitive mechanism. The authors suggested that the effects of ketanserin may be due to the drug binding non-specifically to the 5-HT transporter, or that 5-HT may regulate the activity of the transporter via an action at the 5-HT_2 receptor (Koch & Galloway, 1997). Crespi *et al.* (1997) reported that MDMA and *p*-chloroamphetamine (PCA) demonstrated equipotent inhibition of [^{3}H]5-HT uptake in rat hippocampal synaptosomes, while PCA was the most potent inducer of [^{3}H]5-HT release, followed by MDMA, fenfluramine and amphetamine (in descending order of potency). Removal of calcium from the incubation medium had no effect on inhibition of uptake, but did result in significant attenuation of MDMA-induced [^{3}H]5-HT release. Thus [^{3}H]5-HT release was shown to be calcium-dependent, while uptake inhibition appeared to be calcium-independent.

(3) 5-HT depletion in regional brain tissue

An acute depletion of 5-HT in the striatum, frontal cortex, hippocampus and hypothalamus has been demonstrated during the first 3 - 4 h after MDMA administration to rats, accompanied by depletion of 5-hydroxyindoleacetic acid (5-HIAA) (see Aguirre *et al.*, 1995; Colado & Green, 1994; Logan *et al.*, 1988; Schmidt,

1987a; 1987b; Schmidt & Kehne, 1990; Schmidt *et al.*, 1986; Stone *et al.*, 1987a; 1987b). Cortical/hippocampal and striatal 5-HT depletion has also been observed in mice (see Logan *et al.*, 1988; O'Shea *et al.*, 2001; Stone *et al.*, 1987a) following MDMA administration.

Colado & Green (1994) administered MDMA (20 mg/kg i.p.) to male Hooded Lister (HL) rats and measured hippocampal and cortical concentrations of 5-HT and 5-HIAA 4 h later. 5-HT was depleted by approximately 80 % in both the hippocampus and cortex, while 5-HIAA was depleted by approximately 60 %. O'Shea *et al.* (2001) administered MDMA to mice (10, 20 or 30 mg/kg i.p., three times at 3 h intervals) and, 3 h after the last injection, demonstrated an acute depletion of 5-HT and 5-HIAA in the hippocampus and cortex, and a decrease in striatal 5-HIAA levels. Stone *et al.* (1987a) demonstrated the differences in MDMA-induced effects on serotonergic activity between rats and mice: 3 h post-injection (rats: 10 mg/kg s.c.; mice: 15 mg/kg s.c.) an approximately 55 % depletion of striatal 5-HT was observed in rats, and an approximately 35 % depletion in mice. At this time-point, an approximately 30 % depletion of 5-HIAA was observed in both species, which, by 6 h, was further diminished in rats but returning towards control values in mice (Stone *et al.*, 1987a). Administration of the 5-HT uptake inhibitor, MDL 27,777 to rats 1 or 2 h after MDMA has been shown to prevent any further decline in hippocampal 5-HT levels, these returning to control values within 6 h after MDMA administration. These data indicate a role for the 5-HT transporter in mediation of MDMA-induced 5-HT release (see Schmidt & Kehne, 1990).

1.2.1.3 Alterations in 5-HT synthesis

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme required for 5-HT synthesis, which has been shown to decrease in activity in response to MDMA administration (see Che *et al.*, 1995; Johnson *et al.*, 1992; Schmidt & Taylor, 1988; Stone *et al.*, 1987a; 1987b; 1988). For example, Stone *et al.* (1987b) demonstrated that TPH activity had started to decline in the neostriatum, frontal cortex, hippocampus and hypothalamus within 15 min after MDMA administration (10 mg/kg s.c.) to male SD rats. This decrease in activity was seen to precede depletion of 5-HT and 5-HIAA. Depletion of central dopamine content (through treatment with α-methyl-*p*-tyrosine (AMPT) or

reserpine), or selective destruction of nigrostriatal dopamine projections (through administration of 6-hydroxydopamine; 6-OH-DA) has been shown to provide partial blockade of MDMA-induced reductions of TPH activity in rats. Thus dopamine appears to be involved in the acute serotonergic effects of MDMA, catecholamine depletion apparently having a protective effect (Stone *et al.*, 1988).

1.2.1.4 Acute release and depletion of dopamine

Acute dopamine release has also been demonstrated, both *in vivo* (see Colado *et al.*, 1999a; Gudelsky *et al.*, 1994; Koch & Galloway, 1997; Nash & Brodkin, 1991; Nixdorf *et al.*, 2001; Sabol & Seiden, 1998; Shankaran & Gudelsky, 1998; 1999; Yamamoto & Spanos, 1988) and *in vitro* (see Crespi *et al.*, 1997; Johnson *et al.*, 1986; O'Loinsigh *et al.*, 2001; Schmidt, 1987b), following MDMA administration to rats. Striatal concentrations of dopamine and its metabolites have also been measured within the first few hours after MDMA administration (see Colado & Green 1994; Gough *et al.*, 1991; Logan *et al.*, 1988; O'Shea *et al.*, 2001; Schmidt *et al.*, 1991; Yamamoto & Spanos, 1988).

(1) In vivo dopamine release

Yamamoto & Spanos (1988) placed *in vivo* voltammetry electrodes in the anterodorsal caudate and nucleus accumbens to enable measurement of dopamine release in awake-behaving rats, following peripheral administration of MDMA (2.5, 5 or 10 mg/kg i.p.). MDMA produced a dose-dependent release of dopamine in both brain areas, being significantly greater in the caudate compared to the nucleus accumbens at the highest dose of MDMA, but of similar magnitude at the two lower doses. The peak release occurred within 120 min after drug administration and returned towards baseline values within 180 min. Colado *et al.* (1997a) administered MDMA (15 mg/kg i.p.) to male DA rats and, using *in vivo* microdialysis, demonstrated a rapid, significant increase in extracellular dopamine concentrations in the striatum, while sustained depletion of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were observed.

The involvement of 5-HT in MDMA-induced acute dopamine depletion has been shown by the fact that fluoxetine pretreatment produces significant attenuation of the response

(Koch & Galloway, 1997), as does pretreatment with the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin (Yamamoto *et al.*, 1995). Gudelsky *et al.* (1994) reported significant potentiation of MDMA-induced striatal dopamine release by pretreatment with either the 5-HT_2 receptor agonist, (±)-2,5-dimethoxy-4-iodoamphetamine (DOI), or the non-selective 5-HT agonist, 5-methoxy-*N,N*-dimethyltryptamine (5-MeODMT). These data indicated that stimulation of 5-HT_2 receptors enhanced dopaminergic activity. Shankaran & Gudelsky (1999) demonstrated that, although MDMA-induced striatal 5-HT release was inhibited by pretreatment with a neurotoxic dose regimen of MDMA, the significant release of dopamine was unaltered. These data indicated that the pretreatment schedule produced selective neurotoxic damage to serotonergic nerve terminals, leaving dopaminergic neurones unaffected.

Yamamoto *et al.* (1995) reported that MDMA administration results in a decrease in extracellular concentrations of γ-aminobutyric acid (GABA) in the substantia nigra, which is prevented by ritanserin infusion (10 μMol). The authors suggested that MDMA-induced striatal dopamine release could be modulated through an interaction between 5-HT and GABA. Shankaran & Gudelsky (1998) demonstrated prevention of an MDMA-induced increase in extracellular dopamine concentrations in the hippocampus by pretreatment with either the noradrenaline uptake inhibitor, desipramine, or *N*-(2-chloroethyl)-*N*-ethyl-2-bromo benzylamine (DSP_4), a compound which is believed to selectively deplete brain noradrenaline. However, neither pretreatment affected MDMA-induced dopamine release within the striatum. These data indicated that MDMA-induced increases in hippocampal extracellular dopamine could occur as a result of dopamine release from noradrenergic nerve terminals, since MDMA taken up into noradrenergic nerve terminals might increase efflux of cytosolic dopamine via the noradrenergic transporter. Thus desipramine could prevent MDMA uptake into the noradrenergic nerve terminal, or prevent dopamine efflux from the terminal (Shankaran & Gudelsky, 1998).

Nash & Brodkin (1991) demonstrated that central administration of MDMA (10 μMol infusion via a microdialysis probe implanted in the striatum) resulted in a pronounced increase in extracellular dopamine concentrations, which was significantly attenuated by

peripheral administration of the selective dopamine uptake inhibitor, 1-[2-*bis* (4-fluorophenyl) methoxy]ethyl]-4-3-phenylpropyl]piperazine (GBR 12909). Dopamine release was also prevented by mazindol, which inhibits uptake of dopamine, noradrenaline and, to a lesser extent, 5-HT. These results indicated that MDMA releases dopamine via an interaction with the dopamine transporter.

(2) In vitro dopamine release

Johnson *et al.* (1986) demonstrated an acute release of [^{3}H]dopamine *in vitro*, following incubation of rat caudate nucleus slices with MDMA (10 μMol), although this response was significantly smaller than the response to MDA administration. Koch & Galloway (1997) demonstrated that incubation of rat striatal slices with MDMA resulted in a dose-dependent release of dopamine, which was completely prevented by pretreatment with GBR 12909, but unaffected by pretreatment with fluoxetine. These data indicated that the dopamine transporter is involved in the mediation of the effects of MDMA, and that intact neurones are likely to be required for 5-HT-facilitated dopamine release, since fluoxetine did not affect *in vitro* dopamine release, but did attenuate *in vivo* release (Koch & Galloway, 1997).

Crespi *et al.* (1997) demonstrated acute [^{3}H]dopamine release in striatal synaptosomes, following incubation with amphetamine, PCA, MDMA and fenfluramine (in descending order of potency). This response was demonstrated to be calcium-dependent, since removal of calcium from the superfusion buffer significantly attenuated [^{3}H]dopamine release. O'Loinsigh *et al.* (2001) demonstrated [^{3}H]dopamine release in striatal synaptosomes as induced by MDMA and its analogues: MDA, MDMA, MDEA and MDBA (in descending order of potency).

(3) Striatal dopamine depletion

Logan *et al.* (1988) measured striatal monoamine concentrations at various time-points following MDMA administration, and reported a rise in dopamine and fall in DOPAC levels, 3 h post-injection in both rats and mice. Yamamoto & Spanos (1988) reported similar results, demonstrating a rise in dopamine levels and fall in DOPAC levels in both the caudate and the nucleus accumbens, which corresponded to the observed

release of dopamine as measured via *in vivo* voltammetry. Schmidt *et al.* (1991) co-administered MDMA and the 5-HT_2 antagonist, α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11,939) and measured striatal concentrations of dopamine, DOPAC and HVA 3 h later. MDL 11,939 completely blocked MDMA-induced increases in striatal dopamine and significantly potentiated MDMA-induced decreases in DOPAC. While MDMA alone had no effect on striatal HVA concentration, MDL 11,939 + MDMA treatment produced marked depletion of HVA 3 h after MDMA administration. These data indicated a critical role for the 5-HT_2 receptor in the neurochemical effects of MDMA.

O'Shea *et al.* (2001) demonstrated a significant depletion of striatal dopamine in mice, 3 h after the last of three MDMA injections (30 mg/kg i.p.), while striatal DOPAC concentration was dose-dependently depleted (10, 20 or 30 mg/kg i.p., three times at 3 h intervals). Pretreatment with GBR 12909 (30 mg/kg i.p., 30 min prior to each 30 mg/kg MDMA injection) further enhanced MDMA-induced striatal dopamine loss, had no effect on MDMA-induced DOPAC loss, and enhanced striatal HVA concentrations. Fluoxetine pretreatment had no effect on MDMA-induced changes in dopamine. The potentiation of MDMA-induced dopamine loss by GBR 12909 raised the question as to whether this response does in fact involve the dopamine uptake carrier (O'Shea *et al.*, 2001).

1.2.1.5 Free radical production

MDMA administration leads to the production of free radicals, such as hydroxyl radicals ($OH^{\cdot}$) and peroxynitrite radicals ($ONOO^{\cdot}$). The formation of free radicals and the potential involvement of nitric oxide in MDMA-induced neurotoxicity are discussed in Chapter 7. Briefly, Colado *et al.* (1997a) demonstrated the production of free radicals through the use of *in vivo* microdialysis in the hippocampus of male DA rats. An *in vivo* system can be used to measure the production of $OH^{\cdot}$, whereby salicylic acid is perfused through the microdialysis probe and, when $OH^{\cdot}$ reacts with the salicylate, 2,3- and 2,5-dihydroxybenzoic acid (2,3-DHBA and 2,5-DHBA) are generated. Thus by measuring the production of 2,3-DHBA and 2,5-DHBA, $OH^{\cdot}$ generation can be assessed. MDMA (15 mg/kg i.p.) was shown to produce a sustained (50 %) rise in the dialysate

concentration of 2,3-DHBA, a response which was prevented by administration of the spin trap reagent, PBN (120 mg/kg i.p., 10 min prior to and 2 h after MDMA). These data thus demonstrated the production of free radicals following MDMA treatment, and that the protective action of PBN is likely to involve a free radical scavenging mechanism.

1.2.1.6 Acute behavioural effects

(1) Serotonin behavioural syndrome and locomotor activity

The "serotonin behavioural syndrome" was first reported in rats by Grahame-Smith (1971) within 30 min after administration of the monoamine oxidase (MAO) inhibitor, tranylcypromine (TCP). Hyperactivity was accompanied by head-weaving, piloerection, forepaw treading, proptosis, penile erection, ejaculation, salivation and defecation. The time of onset of hyperactivity was decreased with increasing dose of L-tryptophan, which was administered 30 min after TCP. Administration of MDMA to rats (and mice, see Miller & O'Callaghan, 1995) results in an acute, dose-dependent, hyperlocomotor response (Callaway *et al.*, 1990; Colado *et al.*, 1993; Dafters, 1994; De Souza *et al.*, 1997; Kehne *et al.*, 1996a; Marston *et al.*, 1999; McCreary *et al.*, 1999; McNamara *et al.*, 1995; O'Loinsigh *et al.*, 2001; Slikker Jr. *et al.*, 1989; Spanos & Yamamoto, 1989), which commences within 30 min after drug injection (Spanos & Yamamoto, 1989). In addition, serotonin syndrome behaviour is observed, which comprises: forepaw treading, head-weaving, low body posture, piloerection and salivation (Colado *et al.*, 1993; De Souza *et al.*, 1997; Marston *et al.*, 1999; Shankaran & Gudelsky, 1999; Slikker Jr. *et al.*, 1989; Spanos & Yamamoto, 1989).

Slikker Jr. *et al.* (1989) administered MDMA (5 or 10 mg/kg p.o.) to male SD rats, once per day on four consecutive days, and assessed spontaneous behaviour for 60 min commencing 10 min after each dose. Locomotor activity and serotonin syndrome behaviours were monitored and scored during 20, 15 s observation periods. On the first day, the serotonin motor syndrome score was significantly higher in MDMA-treated animals compared to controls, however on the other three test days, serotonin syndrome scores decreased on each successive day and were no different from control values. Overall locomotor activity was also greater in MDMA-treated animals on the first test

day, although this did not reach statistical significance. There were no significant differences between MDMA-treated and control animals on any of the subsequent test days. The authors suggested that the decrease in serotonin motor syndrome scores on the later test days were likely to be due to MDMA-induced serotonergic deficits (Slikker Jr., 1989). Callaway *et al.* (1990) demonstrated a dose-related effect of MDMA on locomotor activity, the greatest locomotor scores being obtained after administration of the highest two doses (3 and 10 mg/kg s.c., 10 min prior to testing). Control and MDMA-treated animals administered the lower doses of drug (0.3 and 1 mg/kg s.c.) demonstrated a decrease in activity over time, while animals administered the highest two doses of MDMA demonstrated an increase in activity which peaked 30 min after drug administration and was still markedly greater than the lower dose and control groups at the end of the test. Fluoxetine pretreatment prevented the MDMA-induced increase in locomotor activity, indicating that 5-HT release plays an important role in the behavioural effects of MDMA.

MDMA-induced locomotor responses have been shown to be, at least partially, mediated by the 5-HT_{1B} receptor. For example, McCreary *et al.* (1999) demonstrated MDMA-induced hyperactivity in rats in an open-field/activity meter apparatus, which was blocked by pretreatment with the 5-$HT_{1B/1D}$ receptor antagonist, 2'-methyl-4'-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide (GR 127935), while the 5-HT_{1A} antagonist, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide (WAY 100,635) had no effect. Scearce-Levie *et al.* (1999) administered MDMA (3.3, 10 or 30 mg/kg i.p.) to wild-type and 5-HT_{1B}-knockout mice, 10 min prior to analysis of locomotor behaviour in an open field arena. The lowest dose had no effect on locomotor activity in either group, whereas 10 mg/kg and 30 mg/kg doses resulted in two-fold and four-fold increases in locomotor activity, respectively, in wild-type mice. Only the highest dose produced an increase in locomotor activity in the knockout mice, although this response was delayed - the wild-type mice demonstrated significantly greater locomotor activity during the period 0 - 30 min, while there was no difference between groups during the period 60 - 90 min. The alterations in MDMA-induced locomotor behaviour were confirmed to be due to the absence of the 5-HT_{1B} receptor, since

administration of the 5-$HT_{1B/1D}$ antagonist, GR 127935, blocked MDMA-induced locomotor stimulation in a similar manner to that observed in the knockout mice. The 5-HT_{2A} receptor also appears to be involved in expression of the MDMA-induced locomotor response. Kehne *et al.* (1996a) demonstrated a reduction of the response by pretreatment with the 5-HT_{2A} receptor antagonist, R-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol (MDL 100,907), while MDMA-induced increases in rearing behaviour were unaffected. This reduction was greater in the latter portion of the test (30 - 60 min) than the earlier portion (0 - 30 min).

(2) Anxiety-related behaviours

The anxiety-related responses which occur following acute MDMA administration are discussed in Chapter 4. In particular, Lin *et al.* (1999) reported dose-dependent effects, as measured on the elevated plus-maze 30 min after MDMA administration to mice; anxiogenic effects were observed at lower doses and anxiolytic effects at higher doses, shown by changes in the percentage (%) number of open arm entries and time spent on the open arms. In rats, Morley & McGregor (2000) demonstrated a dose-related decrease in the time spent on the open arms and the total number of arm entries, indicating an anxiogenic effect at these doses (1.25 - 5 mg/kg). Maldonado & Navarro (2001) conducted a study on social interaction behaviours between male mice, 30 min after MDMA injection (1, 8 or 15 mg/kg i.p.). MDMA-treated animals performed significantly less grooming, digging, social investigation (sniffing, grooming, crawling over or under paired animal), threat (sideways or upright offensive) and attack (charge, lunge, chase) behaviours compared to control animals. Non-social exploration (rearing, exploring), defence/submission (upright defensive, upright submissive), exploration from a distance (stretched attend posture) and avoidance/flee (retreat, flinch, startle) behaviours were all increased in MDMA-treated mice. These behavioural changes are indicative of anxiogenic-like activity in MDMA-treated mice (Maldonado & Navarro, 2001).

1.2.2 Long-term effects

The long-term effects which follow MDMA administration to rats include: (1) a depletion of regional brain 5-HT, (2) neuronal damage and a reduction of 5-HT uptake

sites, (3) alterations in 5-HT receptor density, (4) behavioural changes. 5-HT depletion has also been demonstrated in guinea-pigs, and both loss of 5-HT content and serotonergic neuronal damage have been reported in non-human primates, whereas MDMA administration to mice results in long-term depletion of brain dopamine.

1.2.2.1 Long-term 5-HT depletion and neuronal damage in rats

(1) Effects of preventing MDMA-induced acute hyperthermia

Prevention of the MDMA-induced hyperthermic response tends to provide protection against loss of 5-HT and its metabolite (see Chapter 7) and a number of compounds which were previously shown to be neuroprotective have later been demonstrated to induce hypothermia or, at least, prevent hyperthermia. For example, Farfel & Seiden (1995a) demonstrated that the NMDA antagonist, dizocilpine (MK-801), protected against MDMA-induced depletions of 5-HT and 5-HIAA measured three days post-treatment. However, co-administration of MK-801 and MDMA was also demonstrated to produce a hypothermic response. When the temperatures of MK-801 + MDMA animals were kept elevated, the neuroprotective effects of MK-801 were completely reversed. Similar effects were seen when the competitive NMDA receptor antagonist, *cis*-4-(phosphonomethyl)-2-piperidine carboxylic acid (CGS 19755) was co-administered with MDMA - both a hypothermic response and some degree of protection against serotonergic depletion were observed. However, pretreatment with the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), did not modify the MDMA-induced hyperthermic response, and did not protect against 5-HT and 5-HIAA depletion (Farfel & Seiden, 1995a).

Malberg *et al.* (1996) investigated the effects of three pretreatment compounds on MDMA-induced hyperthermia and serotonergic neurotoxicity in male Holtzman rats: (1) ketanserin, (2) the competitive tyrosine hydroxylase inhibitor, AMPT, and (3) fluoxetine. Pretreatment with either ketanserin (6 mg/kg i.p.) or AMPT (75 mg/kg i.p.) resulted in a hypothermic response to MDMA (40 mg/kg s.c.) and, two weeks later, prevented MDMA-induced reductions in brain 5-HT and 5-HIAA concentrations. In order to prevent the hypothermic response to ketanserin/MDMA and AMPT/MDMA,

another group of animals were warmed (via a heating lamp positioned above their cage) to maintain core temperatures of 38.5 - 39.5 °C. This procedure abolished the neuroprotective effects of ketanserin and AMPT; 5-HT and 5-HIAA concentrations being similar to those seen in MDMA-treated animals. Fluoxetine pretreatment (10 mg/kg i.p.) did not significantly modify the MDMA-induced hyperthermic response, but did protect against 5-HT and 5-HIAA depletion. Thus, these results indicated that prevention of MDMA-induced hyperthermia, by ketanserin and AMPT, provided neuroprotection against loss of 5-HT and 5-HIAA. The neuroprotective effects of fluoxetine (which was without effect on MDMA-induced hyperthermia) indicated that protection was afforded by some mechanism other than temperature modification. The authors suggested that selective blockade of the serotonin transporter by fluoxetine might prevent the uptake of MDMA or putative neurotoxins into the nerve terminal, thus providing a protective mechanism against MDMA-induced neurotoxicity (Malberg *et al.*, 1996).

Malberg & Seiden (1998) demonstrated the apparent importance of T_a in depletion of 5-HT and 5-HIAA, as measured two weeks after MDMA administration (20 or 40 mg/kg s.c.). No significant depletions were observed in any of the brain regions examined, where MDMA had been administered at a T_a of 20, 22 or 24 °C. However, under T_a conditions of 26, 28 or 30 °C, significant depletions of both 5-HT and 5-HIAA were observed in the hippocampus, striatum, frontal cortex and somatosensory cortex. Significant negative correlations between core temperature and serotonergic depletion were observed. Thus small changes in T_a were shown to produce marked changes in the degree of serotonergic neurotoxicity.

(2) 5-HT depletion in rats and guinea-pigs

Administration of single or multiple doses of MDMA to rats results in long-term depletions of 5-HT and 5-HIAA (see Aguirre *et al.*, 1998; Battaglia *et al.*, 1987; Colado *et al.*, 1993; 1999a; Commins *et al.*, 1987; Farfel & Seiden, 1995a; Gudelsky, 1996; Malberg *et al.*, 1996; Nash & Yamamoto, 1992; O'Loinsigh *et al.*, 2001; O'Shea *et al.*, 1998; Sanchez *et al.*, 2001; Scanzello *et al.*, 1993; Schmidt, 1987a; 1987b; Shankaran &

Gudelsky, 1998; 1999) and significant reductions in TPH activity (see Schmidt *et al.*, 1990c; Stone *et al.*, 1987a; 1987b; 1988; Yeh, 1999).

Following the initial decrease in 5-HT content, as 5-HT is released, concentrations return towards pretreatment levels within 24 h. Schmidt (1987a) administered a single dose of MDMA (10 mg/kg s.c.) to male SD rats and monitored the time course of cortical 5-HT depletion, showing two clearly distinguishable phases of the response. 5-HT was significantly depleted within 3 h of drug treatment, being only 16 % of control values at between 3 h and 6 h post-drug administration. Between 6 h and 24 h, however, a sharp recovery was observed; 5-HT concentration had returned to control values one day after MDMA injection. The second phase of depletion was apparent one week post-treatment, as 5-HT levels gradually declined during the period between one and seven days, being reduced to 74 % of control values one week after drug administration. Battaglia *et al.* (1988a) demonstrated dose-dependent reductions in frontal cortex concentrations of 5-HT and 5-HIAA in male SD rats during the sub-acute phase, 18 h after multiple doses of MDMA (5, 10 or 20 mg/kg s.c. twice per day for four consecutive days). Following the highest dose, 5-HT and 5-HIAA were reduced by approximately 85 % and 60 %, respectively. Similar reductions were observed following four 20 mg/kg doses administered to guinea-pigs. In addition, the loss of 5-HT and 5-HIAA content were shown to be dependent upon the number of doses of MDMA (10mg /kg) administered - 5-HT was reduced by approximately 10 %, 20 %, 40 % and 80 % following one, two, four or eight doses, respectively (Battaglia *et al.*, 1988a). Similar results were reported by Stone *et al.* (1986) 18 h after MDMA administration (10 mg/kg s.c., five times at 6 h intervals), in addition to significant reductions in TPH activity.

Following the acute/sub-acute depletion of 5-HT, long-term depletion occurs, is apparent within four days after drug administration and persists for many weeks. O'Shea *et al.* (1998) conducted a comprehensive study on MDMA-induced long-term serotonergic depletion, assessing the extent of neurotoxicity produced by: (1) single doses of MDMA (4, 10 and 15 mg/kg i.p.), (2) multiple low doses (4 mg/kg) administered once or twice daily for four consecutive days, and (3) multiple low doses

(4 mg/kg) administered twice weekly for eight consecutive weeks. Single doses produced dose-dependent decreases in hippocampal, cortical and striatal 5-HT and 5-HIAA measures one week post-treatment, the lowest dose (4 mg/kg) only having a significant depleting effect on cortical 5-HIAA. Administration of 4 mg/kg MDMA daily for four days had no effect on regional brain concentrations of 5-HT or 5-HIAA, while twice daily administration resulted in significant depletions in all brain areas examined (e.g. 40% cortical loss of 5-HT). Twice weekly administration of low dose MDMA had no effect on regional brain concentrations of 5-HT or 5-HIAA. The authors suggested that, since MDMA-induced damage is likely to involve free radicals, the absence of serotonergic depletion following chronic, low dose MDMA could be due to the recovery of endogenous free radical scavenging systems between each administration. In addition, the data indicated that high or frequent doses of MDMA produced neurotoxic damage and could have significant implications for the recreational use of MDMA (O'Shea *et al.*, 1998).

Colado *et al.* (1993) reported significant depletion of both 5-HT and 5-HIAA in the cortex and hippocampus four days after a single dose of MDMA (20 mg/kg i.p.). Administration of the GABAmimetic compound, clomethiazole (100 mg/kg i.p., 20 min after MDMA injection), significantly attenuated or prevented 5-HT and 5-HIAA loss in both brain regions. Administration of clomethiazole (50 mg/kg i.p.) pre- and post-MDMA injection afforded complete protection against MDMA-induced loss of 5-HT and 5-HIAA in both brain regions, whereas MK-801 treatment (1 mg/kg i.p., 5 min before and 55 min after MDMA) only prevented 5-HT and 5-HIAA loss in the hippocampus. Since MK-801 is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, these data could indicate that MDMA-induced neurotoxicity involved excitotoxic damage (Colado *et al.*, 1993). This study was further extended (Colado & Green, 1994) to investigate the effects of another GABA-potentiating compound, pentobarbitone, and two drugs which inhibit dopamine release in the striatum (γ-butyrolactone; GBL) and nucleus accumbens (ondansetron), on long-term 5-HT depletion. Both pentobarbitone and GBL provided substantial protection against MDMA-induced 5-HT and 5-HIAA depletion in the hippocampus and cortex, with the exception of loss of cortical 5-HIAA which was not prevented by pentobarbitone. These

data thus indicated that MDMA-induced neurotoxicity might also involve striatal dopamine release, since GBL provided protection against MDMA-induced serotonergic depletion. None of the pretreatment compounds had any effect on the acute release of 5-HT induced by MDMA (4 h post-injection), thus it appears that their neuroprotective actions do not involve prevention of 5-HT release (Colado & Green, 1994).

The protective effects of clomethiazole were later demonstrated to partially involve prevention of the MDMA-induced hyperthermia, and partly some other unknown mechanism. Colado *et al.* (1998) demonstrated complete protection against MDMA-induced reductions in hippocampal, cortical and striatal 5-HT and 5-HIAA, by administration of clomethiazole measured seven days post-treatment. However, the acute hyperthermic response to MDMA administration was completely prevented by clomethiazole treatment, thus a second experiment was performed where the animals were warmed via a homeothermic blanket, to maintain their body temperature at similar values to those observed in animals administered MDMA alone. The clomethiazole-induced prevention of hyperthermia was blocked and the neuroprotective effects of clomethiazole were diminished, but not prevented. Thus the authors suggested that some additional mechanism(s) were involved in the neuroprotective effects of clomethiazole (Colado *et al.*, 1998). Such mechanisms have been shown not to include free radical scavenging, as clomethiazole was shown only to prevent free radical production (as indicated by MDMA-induced increases in 2,3-DHBA generation from salicylic acid) where the acute hyperthermic response was prevented. When clomethiazole + MDMA animals were warmed to maintain body temperature similar to that of MDMA-treated animals, inhibition of MDMA-induced increases in 2,3-DHBA generation were prevented. Furthermore, the protection against long-term 5-HT depletion was still afforded by clomethiazole, even when free radical production was not inhibited (Colado *et al.*, 1999b).

Seven days after a single dose of MDMA (10 mg/kg i.p.) was administered to male DA rats, cortical and hippocampal 5-HT concentrations were both reduced by 40 %. The depletion of both 5-HT and 5-HIAA was significantly attenuated by pretreatment with the spin trap reagent, α-phenyl-N-tert-butyl nitrone (PBN; 150 mg/kg i.p., 10 min prior

to and 2 h after MDMA), 5-HT depletion being only 16 % and 13 % in the cortex and hippocampus, respectively in the PBN + MDMA treatment group (Colado & Green, 1995). Similar results were reported by Yeh (1999), where PBN attenuated both MDMA-induced 5-HT and 5-HIAA depletion, and MDMA-induced decreases in [^{3}H]paroxetine binding. However, PBN was also demonstrated to prevent the MDMA-induced hyperthermic response, which may have contributed to the protective effect of PBN (Colado & Green, 1995; Yeh, 1999). While the protective effects of PBN could involve attenuation of the acute hyperthermic response, the involvement of free radicals in MDMA-induced neurotoxicity has been illustrated by the protective effects of the anti-oxidants, ascorbate and cysteine. Gudelsky (1996) administered MDMA (20 mg/kg s.c.) to male SD rats, which resulted in significant reductions of striatal 5-HT one week later. However, in animals which were administered sodium ascorbate (250 mg/kg i.p.) or cysteine (500 mg/kg i.p.) 30 min prior to and 5 h after MDMA, significant attenuation of MDMA-induced 5-HT loss was observed. Thus these data are indicative of the involvement of oxidative damage in MDMA-induced serotonergic depletion.

Schmidt *et al.* (1990a; 1990b; 1991) demonstrated the protective effects of 5-HT_2 receptor antagonists on MDMA-induced serotonergic depletion. Both MDL 11,939 and ritanserin significantly attenuated MDMA-induced 5-HT depletion, measured one week post-treatment (Schmidt *et al.*, 1990a; 1990b). However, partial attenuation of the acute hyperthermic response was also observed following MDL 11,939 administration which could account for its protective effects. MDL 11,939 was also shown to block MDMA-mediated stimulation of dopamine synthesis and was thus believed to prevent the maintenance of MDMA-induced, carrier-mediated dopamine release, indicating that such dopamine release is required for MDMA-induced serotonergic neurotoxicity (Schmidt *et al.*, 1990b). This suggestion is also consistent with the observations that antagonism of MDMA-induced neurotoxicity can be achieved by: (1) prior depletion of monoamine vesicular stores via reserpine administration, (2) by depletion of newly synthesised neurotransmitter via decarboxylase inhibition, (3) by inhibition of dopamine synthesis by AMPT (Schmidt *et al.*, 1990c), or (4) by administration of the 5-HT_2 antagonist, MDL 100,907, which inhibits MDMA-induced dopamine release (Schmidt *et al.*, 1992).

The mechanisms involved in the protective effects of fluoxetine have recently been investigated. Sanchez *et al.* (2001) administered MDMA (15 mg/kg i.p.) to male DA rats and measured regional brain concentrations of 5-HT and 5-HIAA and [^{3}H]paroxetine binding of uptake sites one week later. Fluoxetine was administered either: (1) 10 mg/kg i.p., 5 min prior to and 55 min after MDMA administration, or (2) 10 mg/kg i.p., twice, 1 h apart, two, four and seven days prior to MDMA. Acute fluoxetine treatment completely prevented MDMA-induced decreases in 5-HT and 5-HIAA content, and significantly attenuated the decrease in uptake sites. Complete neuroprotection was also observed when fluoxetine was administered two or four days prior to MDMA, and significant protection was seen when fluoxetine was administered seven days prior to MDMA. Acutely, a peak cortical concentration of fluoxetine was recorded 30 min post-administration, which declined rapidly within 24 h, to reach a low, steady concentration four to seven days post-treatment. Concentrations of the main metabolite of fluoxetine, norfluoxetine, reached a peak 48 h after fluoxetine administration, and then declined gradually over the following five days. Thus fluoxetine produces a long-lasting neuroprotection against MDMA-induced serotonergic damage, which is likely to be related to the sustained presence of fluoxetine and norfluoxetine in the brain, both of which are still detectable seven days after fluoxetine administration. Both compounds are selective blockers of the 5-HT transporter, inhibiting 5-HT uptake, and are believed to disrupt the entry of MDMA (or a neurotoxic metabolite) into the presynaptic nerve terminal (Sanchez *et al.*, 2001).

While 5-HT depletion appears to be potentiated by the production of an acute hyperthermia, it is not essential for serotonergic neurotoxicity. For example, Farfel & Seiden (1995a) demonstrated that administration of MDMA (40 mg/kg s.c.) to male SD rats under T_a conditions of 20 - 22 °C, resulted in an acute hypothermic response (a decrease in body temperature of approximately 1 °C) 30 min after injection. Three days later, hippocampal and striatal 5-HT concentrations were decreased by 37 % and 42 %, respectively, similar reductions to those observed in animals where an acute hyperthermic response was produced.

(3) Central administration of MDMA to rats

Serotonergic neurotoxicity has been demonstrated not to occur following central administration of MDMA. For example, Paris & Cunningham (1991) infused MDMA (25 μg/μl) directly into the median- and dorsal- raphe nuclei. Two weeks post-administration, there were no differences in striatal or hippocampal concentrations of 5-HT, dopamine, noradrenaline, or their metabolites. Immunocytochemical analysis also demonstrated no loss of 5-HT cell bodies or non-specific damage, and there were no structural abnormalities (such as inclusion bodies) in the raphe nuclei. Thus intracerebral MDMA injection appears to have no effect on 5-HT neurones, and could indicate that systemic administration and the production of a neurotoxic metabolite are required for neuronal damage.

More recently, Esteban *et al.* (2001) reported that systemic MDMA treatment (15 mg/kg i.p.) resulted in a rapid increase in MDMA concentrations in the hippocampal dialysate (peak concentration of approximately 4.35 μMol, 30 - 60 min post-administration). Central drug administration (100, 200 or 400 μMol perfused through the dialysis probe) produced a dose-dependent increase in the concentration of 5-HT in the hippocampal dialysate, reaching a peak 30 - 60 min post-administration. However, hippocampal 5-HT and 5-HIAA content were no different from control values, when measured seven days later. In addition, central drug administration did not produce an acute hyperthermic response, thus a second group of rats were warmed via a homeothermic blanket to maintain rectal temperature near to that of systemically-treated animals. Seven days later, there was still no evidence of serotonergic depletion. These results were in contrast to those observed following hippocampal perfusion of 5,7-DHT, where a pronounced decrease in 5-HT and 5-HIAA was observed in the ipsilateral hippocampus one week later. Striatal perfusion of MDMA resulted in a rapid increase in extracellular striatal dopamine concentrations, but neither dopamine nor 5-HT concentrations in the striatum were diminished when analysed seven days later. These data further indicated that serotonergic neurotoxicity, following systemic MDMA administration, is likely to be caused by peripherally-formed metabolites.

(4) [^{3}H]paroxetine binding

An indication of serotonergic neuronal damage can be demonstrated by [^{3}H]paroxetine binding to the presynaptic 5-HT transporter - binding is reduced following MDMA administration (see Aguirre *et al.*, 1995; Battaglia *et al.*, 1987; 1988a; 1991; Broening *et al.*, 1995; Colado & Green, 1995; Colado *et al.*, 1995; Hewitt & Green, 1994; Obradovic *et al.*, 1998; O'Loinsigh *et al.*, 2001; O'Shea *et al.*, 1998; Scanzello *et al.*, 1993). For example, seven days after administration of a single dose of MDMA (30 mg/kg i.p.), a 35 % reduction in [^{3}H]paroxetine binding was observed in the frontal cortex, while multiple doses of MDMA (30 mg/kg i.p., twice daily for four consecutive days) resulted in an approximately 45 % reduction (Aguirre *et al.*, 1995). However, dopaminergic and noradrenergic neurones, as investigated by [^{3}H]mazindol binding to dopamine and noradrenaline uptake sites, are unaffected by MDMA administration to the rat (see Battaglia *et al.*, 1987; 1991).

Battaglia *et al.* (1987) demonstrated significant reductions in [^{3}H]paroxetine binding in the cortex, hippocampus, striatum, hypothalamus and midbrain, two weeks after administration of MDMA (20 mg/kg s.c., twice daily for four consecutive days), while the density of dopamine and noradrenaline uptake sites were unaffected, with the single exception of dopamine uptake sites in the midbrain. In the same study, significant loss of 5-HT content was shown in the cortex and hypothalamus, while smaller effects were seen in the striatum and hippocampus, thus being in contrast to the significant reduction of [^{3}H]paroxetine binding in all brain regions examined. These results indicate that using loss of 5-HT content as a measure of neurodegeneration could underestimate the full magnitude of MDMA-induced neurotoxicity (Battaglia *et al.*, 1987). Colado & Green (1995) reported a complete inhibition of MDMA-induced reductions in [^{3}H]paroxetine binding in the cortex, following a single dose of MDMA (10 mg/kg i.p., seven days earlier), by administration of the spin trap reagent, PBN (150 mg/kg i.p., 10 min prior to and 2 h after MDMA). Since [^{3}H]paroxetine binding provides a measure of neuronal degeneration, protection by PBN indicates protection against MDMA-induced neurodegeneration and indicates that free radical production is likely to be involved in the neurodegenerative process (Colado & Green, 1995).

(5) Histological analysis

Silver-staining of rat striatal slices (13 - 16 h after the last of four 80 mg/kg doses of MDMA) demonstrated the presence of argyrophilic deposits in MDMA-treated rats, which were absent in control animals. Primary somatosensory cortex slices contained shrunken, argyrophilic neuronal cell bodies and what appeared to be fragmented dendrites and degenerating axon terminals (Commins *et al.*, 1987). However, the staining method used (Fink-Heimer) does not enable identification of the specific neurotransmitter contained in the damaged nerve terminals, therefore it was not possible to determine whether serotonergic or dopaminergic neurones were affected (see Commins *et al.*, 1987; Molliver *et al.*, 1990; O'Hearn *et al.*, 1988). O'Hearn *et al.* (1988) administered MDMA (20 mg/kg s.c., eight times at 4 h intervals) to male SD rats and performed immunocytochemical analysis of regional brain sections two weeks later. Gross changes, indicated by reduced intensity of staining in MDMA-treated brain slices, were reflective of a marked reduction in serotonergic axonal density and were particularly apparent in the neocortex, striatum and thalamus, with smaller reductions occurring in the hippocampus, septum and amygdala. The terminal portions of axons were shown to be selectively vulnerable to MDMA-induced damage, as indicated by the reduced density of fine, arborised 5-HT axons, while smooth, straight preterminal fibres, fibres of passage and raphe cell bodies were spared. Morphological evidence of damage to axon terminals is consistent with the observed reductions in 5-HT uptake sites (see Molliver *et al.*, 1990; O'Hearn *et al.*, 1988).

The time course of lesion and recovery has been shown to be region-specific - for example, two weeks after drug administration, neurodegenerative processes in the dorsal caudate region are only just fully expressed, while a maximal and persistent deficit in 5-HT innervation is apparent in the cortex, and some regeneration is beginning to occur in the substantia nigra. Furthermore, brain regions containing 5-HT axonal pathways or perikarya are little affected by MDMA, the predominant effects being mediated on axons and terminals (Battaglia *et al.*, 1991). Such data are consistent with the lack of 5-HT depletion in the dorsal raphe region of the brain stem (see Aguirre *et al.*, 1995), which includes serotonergic cell bodies.

(6) Measurement of anterograde axonal transport

Measurement of anterograde axonal transport provides an additional method for assessment of serotonergic neurotoxicity, enabling study of ascending 5-HT axonal projections by tracing the transport of radioactive material. Rats were administered MDMA (20 mg/kg i.p., four times at 2 h intervals), three weeks prior to injection of [^{3}H]proline into the rostral raphe nuclei. Two days after injection of the labelled amino acid, regional brain radioactivity levels were measured. MDMA treatment resulted in significant decreases in anterograde axonal transport of labelled material, which paralleled (but were less severe than) decreases in 5-HT and 5-HIAA content. In addition, the effects of MDMA were similar to those observed following administration of 5,7-DHT, a well-documented serotonergic neurotoxin (Callahan *et al.*, 2001).

(7) Glial fibrillary acidic protein

Astrocyte hypertrophy can occur as a result of neuronal injury and can lead to the enhanced expression of glial fibrillary acidic protein (GFAP). This marker of neuronal damage has been used in assessment of MDMA-induced toxicity, and its use in mice is discussed in Chapter 7. Briefly, striatal GFAP expression has been shown to increase significantly within 72 h of administration of MDMA, MDA and methamphetamine to mice, corresponding to the decrease in striatal dopamine content (see Miller & O'Callaghan, 1994).

1.2.2.2 Alterations in 5-HT receptor density

Aguirre *et al.* (1995) measured *in vitro* binding of [^{3}H]8-OH-DPAT in rat brain cortical and dorsal raphe nucleus homogenates, in order to assess the effects of MDMA administration on 5-HT_{1A} receptor density. Both a single dose (30 mg/kg i.p.) and multiple doses (30 mg/kg i.p., twice daily for four consecutive days) of MDMA resulted in a significant increase in [^{3}H]8-OH-DPAT binding in the frontal cortex, indicating an increase in 5-HT_{1A} postsynaptic receptors in this brain region. However, a single dose had no effect on [^{3}H]8-OH-DPAT binding in the dorsal raphe region, while multiple doses resulted in a significant decrease in binding, indicating a reduction of 5-HT_{1A} inhibitory autoreceptors in this region (Aguirre *et al.*, 1995). [^{3}H]8-OH-DPAT

binding in the hypothalamus has also been shown to be significantly enhanced seven days after either single or multiple doses of MDMA, indicating up-regulation of the 5-HT_{1A} receptor in this brain region (Aguirre *et al.*, 1998). MDMA itself was shown to have negligible *in vitro* affinity for brain 5-HT_{1A} receptors, but increased the density of this receptor subtype in the frontal cortex and reduced receptor density in the dorsal raphe region. A decrease in [^{3}H]paroxetine binding in the frontal cortex correlated with the increase in 5-HT_{1A} receptors, which could indicate adaptive changes to compensate for the loss of serotonergic nerve terminals (Aguirre *et al.*, 1995). Pretreatment with fluoxetine, haloperidol or ketanserin prevented MDMA-induced increases in [^{3}H]8-OH-DPAT binding in the frontal cortex (Aguirre *et al.*, 1998).

While 5-HT_{1A} receptors appear to be up-regulated following MDMA administration, a transient down-regulation of 5-HT_2 receptors has been reported. Scheffel *et al.* (1992) performed *in vivo* and *in vitro* labelling of 5-HT_2 and 5-HT_{1C} receptors in rat brain using the radioligand *N* 1-methyl-2-[^{125}I]lysergic acid diethylamide ([^{125}I]MIL). *In vivo* [^{125}I]MIL binding was unaffected by acute MDMA administration (20 mg/kg s.c.), whereas chronic administration (20 mg/kg s.c., twice daily for four days) resulted in significant decreases in binding (a 55 - 80 % decrease in specific binding was observed 24 h post-treatment). However, seven days and 21 days after cessation of treatment, there were no differences between MDMA-treated and control groups with respect to specific binding of [^{125}I]MIL. Autoradiographic studies confirmed reductions in ([^{125}I]MIL binding in the claustrum, frontal cortex, parietal cortex and striatum, while no effects of MDMA were observed in the choroid plexus, thus 5-HT_{1C} receptors appeared unaffected by MDMA treatment.

1.2.2.3 Recovery of 5-HT content, [^{3}H] paroxetine-labelled uptake sites, axonal morphology and synaptosomal [^{3}H]5-HT uptake in rats

The extent of recovery of 5-HT content, uptake sites, synaptosomal [^{3}H] uptake and axonal morphology in rats (see Lew *et al.*, 1996; Sabol *et al.*, 1996; Scanzello *et al.*, 1993) have been investigated up to one year after MDMA administration. For example, Scanzello *et al.* (1993) reported that the earliest recovery of 5-HT content was observed in the hypothalamus, eight weeks after MDMA administration (10 mg/kg i.p., four

times at 2 h intervals), while hippocampal and striatal levels had recovered by 16 weeks. All brain regions examined showed complete recovery of 5-HT within one year of drug treatment, and similar patterns were observed in the recovery of 5-HIAA content. [^{3}H]paroxetine binding values in the cortex and striatum had returned to control levels within 32 weeks, while hippocampal binding was still 29 % below control values at 52 weeks post-treatment. However, with regard to morphological changes, only one of three animals demonstrated recovery 52 weeks post-treatment; the other two animals still showed marked reductions in axon density.

Sabol *et al.* (1996) demonstrated that MDMA administration (20 mg/kg s.c., twice daily for four consecutive days) resulted in a significant decrease in synaptosomal [^{3}H]5-HT uptake two and eight weeks after treatment, but there was no difference between MDMA- and saline-pretreated animals at any of the later time-points. In contrast to the results of Scanzello *et al.* (1993), significant depletion of 5-HT was still apparent in both the frontal-parietal- and occipital-temporal- cortex one year post-treatment, while some hyperinnervation was observed in the hypothalamus at 52 weeks, compared to control animals. Thus these data showed that MDMA-induced 5-HT depletion and rate of recovery are region-dependent (Sabol *et al.*, 1996). In an accompanying study, Lew *et al.* (1996) reported that 5-HT uptake site density in the frontal-parietal cortex (as measured by ^{125}I-RTI-55 binding) was reduced by 75 % two weeks post-drug administration. Partial recovery was observed at 16 weeks (53 % below control values), but the uptake site density at 52 weeks was still 40 % below control values. The density of hippocampal sites was significantly reduced two weeks post-treatment (66 % below control values), partial recovery being apparent by 16 weeks, and full recovery by 52 weeks post-treatment.

1.2.2.4 Long-term 5-HT depletion and neuronal damage in non-human primates

(1) 5-HT depletion and neuronal damage

Serotonergic depletion and neuronal damage has also been demonstrated in non-human primates (see Fischer *et al.*, 1995; Hatzidimitriou *et al.*, 1999; Insel *et al.*, 1989; Ricaurte & McCann, 1992; Ricaurte *et al.*, 1988a; 1988b; 1992; Scheffel *et al.*, 1998;

Slikker Jr. *et al.*, 1988; 1989; Wilson *et al.*, 1989), the effects being more pronounced than those observed in rodents. For example, administration of MDMA to rats and squirrel monkeys (twice daily, for four consecutive days), illustrated the significantly greater sensitivity of monkeys to the 5-HT depleting effects of MDMA. The dose-response curve was considerably steeper in monkeys and the maximal effect was greater in the monkey than the rat (Ricaurte & McCann, 1992; Ricaurte *et al.*, 1988b).

The degree of 5-HT depletion is dependent upon the route of administration - oral administration results in lower levels of depletion than subcutaneous injection (see Ricaurte *et al.*, 1988c). Since human recreational users of MDMA tend to ingest single doses several days or weeks apart, a single dose of MDMA (5 mg/kg p.o.) was administered to squirrel monkeys and 5-HT levels measured two weeks later. While multiple doses resulted in significant losses in all brain areas examined, a single dose only produced significant depletion of 5-HT in the thalamus (20 %) and hypothalamus (16 %) (Ricaurte *et al.*, 1988c). However, since a single 5 mg/kg oral dose is equivalent to a 1.4 mg/kg dose in a 70 kg human, based on interspecies dose scaling (see McCann & Ricaurte, 2001), and is thus within the same range as a recreational dose (100 - 150 mg tablet), these data indicate a risk of serotonergic damage in humans even after a single dose (see Ricaurte *et al.*, 1988c).

In addition to reductions in 5-HT content, reduced cerebrospinal fluid (CSF) concentrations of 5-HIAA have also been demonstrated following MDMA administration to squirrel- (Ricaurte *et al.*, 1988a) and Rhesus- monkeys (Insel *et al.*, 1989). These data suggested that measurement of CSF 5-HIAA could be used to detect serotonergic damage in living primates, and provided a potential basis for evaluating the effects of recreational MDMA use in humans (Ricaurte *et al.*, 1988a). Abnormal brain 5-HT innervation patterns have still been shown seven years after MDMA administration to squirrel monkeys (5 mg/kg s.c., twice daily for four days). Hatzidimitriou *et al.* (1999) reported that 5-HT axon density remained decreased (approximately 50 - 65 % of control values), in all neocortical regions examined, seven years post-treatment. The caudate and putamen demonstrated partial recovery, while the globus pallidus showed some evidence of hyperinnervation.

(2) In vivo detection of MDMA-induced neurotoxicity

Scheffel *et al.* (1998) investigated the effects chronic MDMA treatment (5 mg/kg s.c. twice daily for four consecutive days) in the baboon, using positron emission tomography (PET) and a selective 5-HT transporter radioligand, [^{11}C](+)McN5652. Significant reductions in regional radioactivity were apparent in the hypothalamus and frontal cortex of MDMA-treated animals 13 days post-treatment, and decreases in [^{11}C](+)McN5652 accumulation occurred in all regions examined during the first 40 days post-treatment. Nine months after MDMA administration, significant recovery of [^{11}C](+)McN5652 binding was observed in the midbrain and hypothalamus, while persistent reduction were seen in all cortical regions. At 13 months, [^{11}C](+)McN5652 concentrations were approximately 35 % greater than control levels in the pons, midbrain and hypothalamus, whereas tracer concentrations remained diminished in all cortical areas. Thus a time-dependent redistribution of 5-HT transporter sites was demonstrated in the baboon brain, which is likely to reflect the differential recovery of 5-HT axon projections. In addition, the study demonstrated a potential method for performing a similar study in human recreational users of MDMA.

1.2.2.5 Long-term dopamine depletion in mice

While 5-HT is selectively depleted following MDMA administration to rats, guinea-pigs and non-human primates, dopamine is selectively lost in mice (see Chapter 7). For example, Battaglia *et al.* (1988a) clearly demonstrated the lack of serotonergic neurotoxicity in mice, as administration of MDMA (20 mg/kg s.c. twice per day for four consecutive days) resulted in significant loss of 5-HT and 5-HIAA content and 5-HT uptake sites in the frontal cortex of both rats and guinea-pigs, whereas none of these parameters were altered in the mouse. Logan *et al.* (1988) demonstrated an initial decline in mouse cortex/hippocampus 5-HT concentrations (three days post-treatment), which had returned to control values within two weeks of drug administration. Striatal dopamine and DOPAC concentrations, however, were depleted by approximately 50 %, three days post-treatment, and demonstrated no recovery within two weeks of drug administration.

However, while long-term depletion of dopamine has not been shown in rats following MDMA administration, an acute release of dopamine has been reported (see above), thus dopamine could be involved in the production of MDMA-induced serotonergic neurotoxicity. Colado *et al.* (1999a) demonstrated that administration of the dopamine precursor, L-DOPA (25 mg/kg i.p., 2 h after MDMA administration), prolonged the MDMA-induced hyperthermic response and increased the acute release of striatal dopamine. However, L-DOPA had no effect on either the acute production of free radicals, or the 5-HT depletion measured seven days post-drug administration, thus it appears that acute dopaminergic effects are not involved in long-term neurotoxicity in rats.

1.2.2.6 Long-term behavioural changes

Shankaran & Gudelsky (1999) reported a significant reduction in MDMA-induced serotonin syndrome behaviours in rats which had previously been treated with a neurotoxic dose regimen (10 mg/kg i.p., four times at 2 h intervals), compared to rats administered a single dose of MDMA (7.5 mg/kg i.p.). The authors suggested that these data reflected long-term functional consequences of MDMA administration, and were indicative of 5-HT depletion resulting from the multiple dose regimen. Spanos & Yamamoto (1989) showed that chronic administration of MDMA (2, 5 or 7.5 mg/kg i.p., on alternate days, up to a total of 12 doses) had no long-term effects on locomotor activity, when assessed 48 h after the last dose. However, the intensity of all serotonin syndrome behaviours increased in a dose-dependent manner following chronic drug administration.

McNamara *et al.* (1995) measured locomotor activity in an open field arena on each day of MDMA administration (5, 10 or 20 mg/kg i.p., twice daily for four consecutive days) and on the four days following the treatment period. While total locomotor activity was significantly higher in MDMA-treated rats (administered 10 or 20 mg/kg) compared to control animals during the drug treatment period, activity had returned to baseline/control values within 48 h after the last drug administration. Thus, using this treatment regimen, the MDMA-induced increase in locomotor activity was dose- and time-dependent and returned to normal following cessation of drug treatment. In

contrast, Wallace *et al.* (2001) reported reductions in locomotor activity, one week after multiple doses of MDMA (10 mg/kg i.p., four times at 2 h intervals). Spontaneous locomotor activity was measured during diurnal and nocturnal cycles for seven consecutive days, and MDMA-treated animals demonstrated significant reductions in activity, compared to control animals, during both cycles. There was no difference in the activity of either treatment group between diurnal and nocturnal values. Such alterations were accompanied by significant reductions in striatal 5-HT levels and, while any connection between the two findings was not established, persistent behavioural modifications had been demonstrated following a monoamine-depleting dose of MDMA.

1.3 EFFECTS OF MDMA IN HUMANS

It is always difficult to make direct comparisons between results obtained in animal studies and those performed in humans, particularly based upon differing metabolic rates and weight:volume ratios of different species; small mammals tend to eliminate drugs at a faster rate than large mammals. However, interspecies scaling enables prediction of drug elimination in different species, based upon the underlying anatomical, physiological and biochemical similarities between most land mammals, and may be calculated by: Y = aWb, where Y = the physiological variable of interest (e.g. heart rate), W = body weight, and log a is the y-intercept and b is the slope obtained from the plot of log Y versus log W (see Mordenti, 1986).

Thus, in order to achieve a similar effect to that seen in humans, smaller animals require higher doses of drug: $D_{human} = D_{animal}\ (W_{human}/W_{animal})^{0.7}$, where D = dose of drug (mg) and W = body weight (kg). So, for example, if a single neurotoxic dose of MDMA administered to a 1 kg monkey were 5 mg/kg, the equivalent dose in a 70 kg human would be:

$5\ (70/1)^{0.7} = 97.8$ mg $\div$ 70 kg = 1.4 mg/kg (McCann & Ricaurte, 2001).

The dose of MDMA (12.5 mg/kg) used in the majority of experiments in rats reported in this thesis would therefore be equivalent to:

$12.5\ (70/0.25)^{0.7} = 645.5$ mg ÷ 70 kg = 9.2 mg/kg.

If a typical MDMA tablet contains 80 mg to 150 mg of MDMA (see Henry, 1992; Schifano, 1991; dancesafe.org; www.drugscope.org.uk; www.erowid.org; www.thesite.org), then 645.5 mg is the equivalent of four to eight tablets. Peroutka (1987) reported that the amount of drug taken in a single dose ranged from 60 - 250 mg (1 - 4 mg per kg body weight), while Bolla *et al.* (1998) stated that, in a sample of 30 MDMA users, an average monthly does was 441 mg (range: 55 - 4000) were ingested per month. Therefore, while 645.5 mg would seem to be a high dose for a single use, it could be ingested by a regular user of MDMA over a period of one to two months. In addition, a 10 mg/kg dose of MDMA administered to male DA rats resulted in a plasma MDMA concentration of 6.3 nMol/ml, 45 min post-injection (Colado *et al.*, 1995). This value is within the same range as has been reported in humans following MDMA ingestion; for example, ingestion of a 150 mg tablet resulted in a plasma concentration of 5.2 nMol/ml (Dowling *et al.*, 1987).

1.3.1 Acute effects

1.3.1.1 Acute physiological effects

The acute adverse physiological effects which occur during the peak period after MDMA ingestion by humans include: elevated blood pressure and heart rate, nausea, chills, diaphoresis, tremor, trismus, bruxism, hyperreflexia, urinary urgency, muscle aches or tension, hot and cold flashes, nystagmus and insomnia (see McCann *et al.*, 1996). One of the major symptoms of MDMA-induced toxicity is hyperthermia, where body temperatures of over 43 °C have been documented and may lead to other toxicological problems during the first few hours following MDMA ingestion, such as: rhabdomyolysis, disseminated intravascular coagulation (DIC) and acute renal failure. Other physiological symptoms which have been reported during the first few hours following ingestion of MDMA, include: tachycardia, coagulopathy, thrombocytopenia, delayed leukocytosis, acidosis, hypoglycaemia, pulmonary congestion, oedema and hepatitis (see Barrett & Taylor, 1993; Brown & Osterloh, 1987; Chadwick *et al.*, 1991; Dowling *et al.*, 1987; Green *et al.*, 1995; Henry *et al.*, 1992; McCann *et al.*, 1996; Milroy *et al.*, 1996; Screaton *et al.*, 1992; Simpson & Rumack, 1981).

1.3.1.2 Acute psychological effects

The acute psychological effects which occur during the peak period following MDMA ingestion by humans include euphoria and reduction of negative thoughts. However, adverse effects may also be experienced, such as depression, irritability, panic attacks, visual hallucinations and paranoid delusions (see Brown & Osterloh, 1987; Creighton *et al.*, 1991; Davison & Parrott, 1997; McCann *et al.*, 1996; Whitaker-Azmitia & Aronson, 1989; www.erowid.org).

1.3.2 Long-term effects

1.3.2.1 Long-term biochemical effects

The longer-term biochemical effects of MDMA use by humans, include: (1) depletion of brain 5-HT and 5-HIAA, (2) reduced density of brain 5-HT transporter sites and receptors, (3) altered central 5-HT function, and (4) alterations in cerebral metabolites (see Chang *et al.*, 1999; Gerra *et al.*, 2000; Kish *et al.*, 2000; McCann *et al.*, 1994; 1998; Price *et al.*, 1989; Reneman *et al.*, 2000a; 2000b; Semple *et al.*, 1999).

(1) Depletion of brain 5-HT and 5-HIAA

Kish *et al.* (2000) reported severe depletion (50 - 80 %) of striatal 5-HT and 5-HIAA in the brain of a 26-year old male, as measured 21 h post-mortem. The subject had taken MDMA regularly for nine years and his use had increased during the three years prior to his death. During this period, he ingested MDMA tablets four or five nights per week, including six to eight tablets taken within a three-day weekend "binge". Post-mortem examination revealed a blood MDMA concentration of 4.4 μg/ml, while concentration in the occipital cortex was approximately 1 μg/g tissue. The subject had also taken cocaine and heroin during the last few months before his death, which might have confounded the reported data. However, the authors stated that neither of these drugs had previously been demonstrated to alter striatal serotonin concentration, therefore they believed that the results seen were most likely to be due to chronic use of MDMA (Kish *et al.*, 2000).

(2) Reduced density of brain 5-HT transporter sites and 5-HT receptors

McCann *et al.* (1998) and Ricaurte *et al.* (2000) employed PET with [^{11}C]McN-5652, in order to assess the extent of brain 5-HT transporter binding in recreational users of MDMA. Experimental subjects had used MDMA on at least 25 occasions, while control subjects had no previous experience with MDMA. The results indicated a lower density of brain 5-HT transporter sites in MDMA users, which positively correlated with the extent of previous MDMA ingestion (McCann *et al.*, 1998; Ricaurte *et al.*, 2000). Semple *et al.* (1999) performed a similar study to McCann *et al.* (1998), whereby single photon emission computed tomography (SPECT) was used with the iodine-123-labelled serotonin transporter ligand, 2β-carbomethoxy-3β-(4-iodophenyl)tropane ([^{123}I]β-CIT). The results indicated a reduction in [^{123}I]β-CIT binding in the MDMA user group, and there was a correlation between the regional uptake of the radioligand and duration of abstinence. The authors suggested that the latter finding might be explained by a reversible down-regulation or occupation of the serotonin transporter (Semple *et al.*, 1999).

(3) Alterations in central 5-HT function

Measurement of the concentration of 5-HIAA in cerebrospinal fluid (CSF) enables an indirect evaluation of central serotonergic function, and has been demonstrated to provide an indication of MDMA-induced neurotoxicity in non-human primates (Ricaurte *et al.*, 1988a). Thirty recreational users of MDMA (on at least 25 occasions) were demonstrated to have significantly lower levels of CSF 5-HIAA compared to control subjects (10.3 ± 3.1 ng/ml and 15.2 ± 7.9 mg/ml, respectively). There was a negative correlation between CSF 5-HIAA and the number of MDMA exposures, although this was not statistically significant, and there were no significant correlations between CSF 5-HIAA and the duration of MDMA use, the frequency of use, or the time elapsed since the last exposure to MDMA (McCann *et al.*, 1994).

(4) Alterations in cerebral metabolites

Chang *et al.* (1999) employed proton magnetic resonance spectroscopy (^{1}H MRS) to measure brain concentrations of N-acetylaspartate (NA), a neuronal marker, and *myo-*

inositol (MI), a tentative glial marker, in order to determine whether MDMA use was associated with changes in neurones and glial cells. Concentrations of creatine (CR), choline compounds (CHO), and glutamate/glutamine (GLX) were also assessed, in addition to metabolite ratios, using CR as internal standard. Initial magnetic resonance imaging (MRI) scans demonstrated no significant brain atrophy or white matter lesions in either MDMA users or control subjects. ^{1}H MRS demonstrated that, in MDMA users, both MI and MI/CR were elevated in the parietal white matter, and CHO/CR was elevated in the occipital grey matter. The duration of MDMA use was correlated to the concentration of MI in parietal white matter and frontal cortex. NA, CR and CHO concentrations were similar in all brain regions examined between MDMA users and control subjects. The elevation of MI concentration indicated increased glial content in the brains of recreational users of MDMA, while the normal NA and GLX concentrations indicated a lack of persistent neuronal damage or ischaemic lesions. The latter could be due to minimal 5-HT neurotoxicity following recreational doses of MDMA (1.5 - 3 mg/kg), or the occurrence of neuronal recovery (Chang *et al.*, 1999).

1.3.2.2 Long-term psychological effects

Longer-term psychological effects resulting from recreational use of MDMA, which can persist after cessation of drug use (see Bolla *et al.*, 1998; Creighton *et al.*, 1991; McCann & Ricaurte, 1991; 1992; McCann *et al.*, 1994; 1996; 1999; McGuire, 2000), include: (1) visual hallucinations and paranoid delusions, which can form part of the peak effects of the drug, but can sometimes persist for days or weeks, (2) anxiety, depression and panic disorder, (3) cognitive impairment, and (4) alterations in behaviour (see Bhattachary & Powell, 2001; Bolla *et al.*, 1998; Creighton *et al.*, 1991; McCann & Ricaurte, 1991; 1992; McCann *et al.*, 1994; 1996; 1999; McGuire & Fahy, 1991; McGuire *et al.*, 1994; McGuire, 2000; Morgan, 1999; 2000; Parrott & Lasky, 1998; Schifano, 1991; dancesafe.org; www.drugscope.org.uk; www.erowid.org; www.thesite.org; www.usdoj.gov/dea).

Alterations in behaviour or personality traits

McCann *et al.* (1994) examined personality traits in a group of recreational MDMA users (see above), and demonstrated that MDMA users showed lower impulsivity and

hostility and greater harm avoidance compared to control subjects. The greatest differences in personality were seen in female MDMA users, who also had the greatest decrement in CSF 5-HIAA. The authors suggested that the nature and regional distribution of MDMA-induced serotonergic deficits might differ from those in patients exhibiting increased impulsivity and aggression (such as individuals with personality disorders, violent offenders or suicidal individuals). In addition, individuals with an inherited MAO-A deficiency, and thus a likely increase in brain serotonin, have been demonstrated to have increased levels of impulsive, aggressive behaviour (see Brunner *et al.*, 1993). Taken together, these data indicate that such personality characteristics are modulated by serotonin (McCann *et al.*, 1994).

McCann *et al.* (1999) compared *m*-CPP-induced changes in behaviour between MDMA users and control subjects, using a series of behavioural assessment scales. In each case, MDMA users demonstrated higher positive scores ("happy", "energetic", "content" and "elated") and lower negative scores ("sad", "tired" and "worried"). MDMA users also demonstrated greater positive- and fewer negative- mood responses to *m*-CPP treatment compared to control subjects. In addition, *m*-CPP treatment can induce panic attacks and, in this study, eight control subjects (32 %) experienced a panic attack compared to one MDMA user (4 %). Thus these data indicated altered behavioural responses to *m*-CPP in recreational users of MDMA, although the authors did note that underlying personality differences in individuals who take MDMA, such as sensation-seeking, could be involved. The authors also suggested that the lowered sensitivity of MDMA users to *m*-CPP-induced anxiety indicated downregulation of postsynaptic 5-HT_{2C} receptors, which are thought to mediate these effects of *m*-CPP (McCann *et al.*, 1999).

1.4 MECHANISMS OF ACTION OF MDMA

MDMA induces the acute release of serotonin and dopamine and its administration results in the long-term depletion of serotonin, in rats, guinea-pigs and non-human primates, and long-term dopamine depletion in mice (see section 1.2). Based on their results obtained in rat hippocampal slices, where MDMA administration resulted in significant [^{3}H]5-HT efflux, Johnson *et al.* (1986) hypothesised that MDMA-induced

[^{3}H]5-HT release could occur via one of three mechanisms: (1) MDMA has a direct postsynaptic receptor-mediated excitatory action, leading to stimulus-coupled 5-HT release at the nerve terminal, (2) MDMA acts indirectly, displacing presynaptic 5-HT stores, by entering the nerve terminal through the 5-HT uptake mechanism, or (3) MDMA acts as a competitive uptake inhibitor, resulting in increased extracellular [^{3}H]5-HT concentrations following spontaneous release (see Johnson *et al.*, 1986; Rudnick & Wall, 1992).

The affinity of MDMA for a range of brain recognition sites has been demonstrated using *in vitro* radioligand binding assays (e.g. [^{3}H]paroxetine, [^{3}H]mazindol and [^{3}H]SCH 23390 binding), MDMA being the most potent at 5-HT uptake sites, with affinity constants in the high nanomolar to low micromolar range. The rank order of affinities at different recognition sites (in descending order of potency) was: 5-HT uptake sites > α_2-adrenoceptors = 5-HT$_2$ receptors = M$_1$ muscarinic receptors = H$_1$ histamine receptors > noradrenaline uptake sites = M$_2$ muscarinic receptors = α_1-adrenoceptors = β-adrenoceptors $\geq$ dopamine uptake sites = 5-HT$_1$ receptors >> D$_2$ dopamine receptors > D$_1$ dopamine receptors (Battaglia *et al.*, 1988b).

The fact that *in vitro* [^{3}H]5-HT release from rat brain slices is calcium-independent indicates that MDMA is unlikely to act via a postsynaptic receptor mechanism (see Johnson *et al.*, 1986). In addition, the likely involvement of the serotonin transporter in MDMA-induced 5-HT release has been demonstrated by the fact that serotonin uptake inhibitors, such as citalopram (see Schmidt *et al.*, 1987) or fluoxetine (see Berger *et al.*, 1992; Gudelsky & Nash, 1996; Mechan *et al.*, 2001b; Shankaran *et al.*, 1999a), prevent *in vivo* and *in vitro* 5-HT release and long-term depletion. For example, Gudelsky & Nash (1996) demonstrated that MDMA-induced increases in extracellular 5-HT concentrations in the striatum were significantly attenuated by fluoxetine pretreatment. The fact that MDMA-induced striatal dopamine release was also attenuated by fluoxetine, indicates that the 5-HT release which results from MDMA administration serves to facilitate dopamine release. The involvement of the serotonin transporter in MDMA-induced 5-HT release has also been demonstrated in rat brain synaptosomes (see Crespi *et al.*, 1997; Fleckenstein *et al.*, 1999; 2000). For example, Crespi *et al.*

(1997) showed that MDMA produced potent inhibition of [^{3}H]5-HT uptake, and high potency for inducing [^{3}H]5-HT release, in rat hippocampal synaptosomes. Inhibition of [^{3}H]dopamine uptake and induction of [^{3}H]dopamine release were also demonstrated in striatal synaptosomes, although MDMA was significantly less potent than amphetamine and PCA in this regard. MDMA-induced inhibition of [^{3}H]5-HT uptake was calcium-independent, while release was, at least partially, inhibited by the removal of calcium ions (Ca^{2+}), indicating calcium-dependence of this mechanism.

Purified platelet plasma membrane vesicles contain the serotonin transporter responsible for 5-HT uptake into presynaptic nerve endings (see Rudnick, 1977), and can therefore be used as a model for investigating the interaction of MDMA with the transporter mechanism. Rudnick & Wall (1992) demonstrated that MDMA produced potent inhibition of the transport of 5-HT into plasma membrane vesicles derived from human platelets. The inhibitory effect of MDMA was suggested to represent competition for the transport site, or could indicate alterations in the ionic gradients involved in 5-HT uptake through permeabilisation of the membrane, for example. However, MDMA was demonstrated to interact directly with the uptake site, due to its ability to displace imipramine, and MDMA-induced 5-HT efflux was shown to require sodium ions (Na^+) on both sides of the membrane. MDMA was also seen to inhibit the ability of chromaffin granule membrane vesicles to accumulate [^{3}H]5-HT and, in addition to a direct effect on the vesicular amine transporter, MDMA was demonstrated to dissipate the transmembrane pH difference (ΔpH). The accumulation of 5-HT is driven and maintained by ΔpH, thus any changes in ΔpH will decrease 5-HT accumulation and increase efflux. The effects of MDMA on ΔpH demonstrate that it is permeant and that it can readily diffuse across membranes (see Rudnick & Wall, 1992; 1993). As MDMA cycles between the cytoplasm and extracellular environment, Na^+ and chloride (Cl^-) ions enter the cell, potassium ions (K^+) leave and hydrogen ions (H^+) remain within the cell, as the protonated form tends to be transported inwards and the neutral form diffuses outwards (Rudnick, 1997; Figure 1.2). The net results of altered ion gradients and increased 5-HT-transporter binding sites, induced by MDMA influx, are 5-HT efflux (Rudnick & Wall, 1992; Rudnick, 1997) and increased intracellular acidity, which may contribute to neurotoxicity (Rudnick, 1997).

MDMA-induced neurotoxicity is likely to involve oxidative stress within the nerve terminal, which could lead to irreversible terminal degeneration (see Rattray, 1991; Figure 1.3). There are a number of possible mechanisms by which toxicity could occur, including:

(1) Metabolism to a quinoid (see Hiramatsu *et al.*, 1990), which can enter the nerve terminal and combine with sulphydryl groups within the active site of TPH.

(2) Redox cycling of MDMA metabolites could generate free radicals, which could lead to inactivation of TPH and damage to protein and lipid components of the nerve terminal (see Rattray, 1991).

(3) Binding of MDMA to the 5-HT transporter or a 5-HT receptor could lead to inactivation of TPH, via increasing intracellular Ca^{2+} concentrations for example (see Azmitia *et al.*, 1990). Elevated Ca^{2+} levels could, in turn, lead to protein kinase C (PKC)-mediated actions, such as proteolysis, and to osmotic stress.

(4) MDMA may inhibit MAO, resulting in oxidation of 5-HT or dopamine to a known neurotoxin, such as 5,7-dihydroxytryptamine (5,7-DHT).

Free radical (hydroxyl radical; $OH^{\cdot}$) generation by MDMA has been demonstrated *in vivo* by quantification of the formation of 2,3-DHBA from salicylic acid (see section 1.2; Colado *et al.*, 1997a; Shankaran *et al.*, 1999a; 1999b; 2001). Shankaran *et al.* (1999a) demonstrated that MDMA-induced production of $OH^{\cdot}$ was completely blocked by pretreatment with fluoxetine. This attenuation could be due to fluoxetine preventing the entry of reactive substances, which can generate free radicals, into the nerve terminal. Shankaran *et al.* (1999b) similarly demonstrated that MDMA-induced $OH^{\cdot}$ production was significantly attenuated by pretreatment with a dopamine uptake inhibitor, mazindol. In addition, mazindol pretreatment prevented MDMA-induced striatal 5-HT depletion, measured seven days post-drug administration. These data indicated that MDMA-induced serotonergic depletion might be due to oxidative stress caused by dopamine-dependent $OH^{\cdot}$ generation, resulting in cytotoxicity.

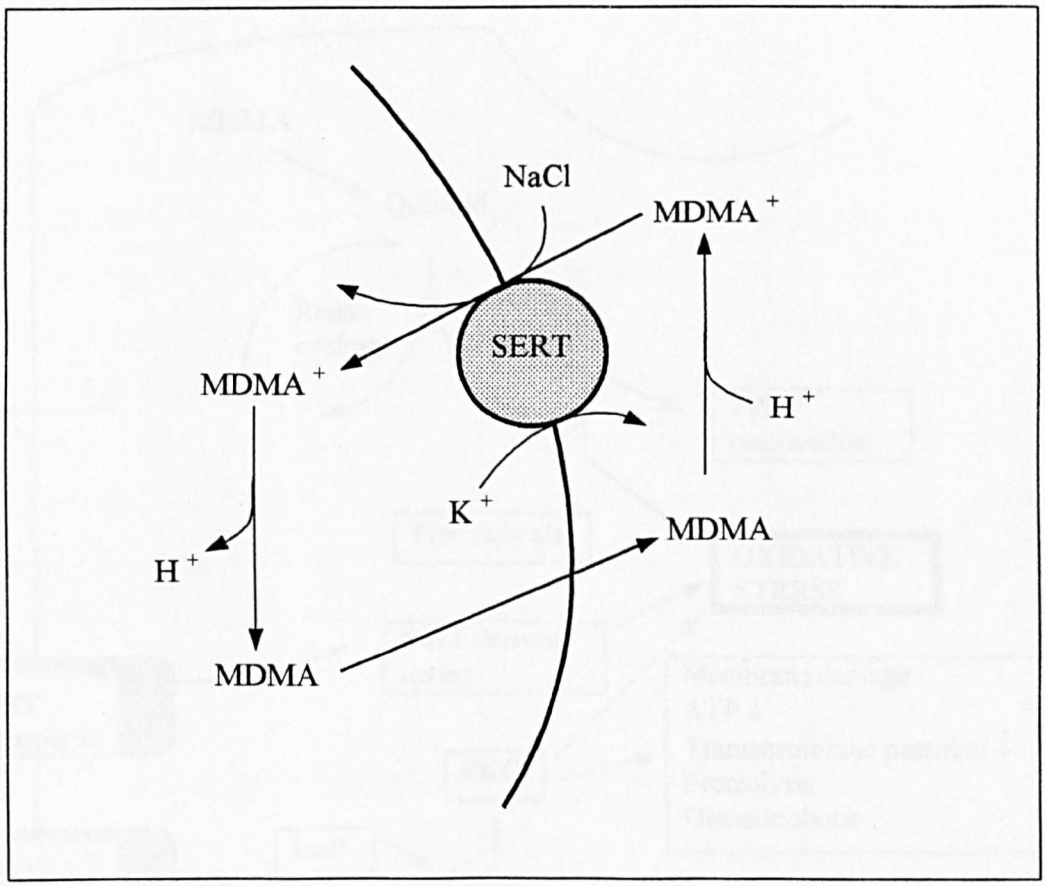

Figure 1.2 Postulated mechanism for the interaction of MDMA with the serotonin transporter (SERT) (after Rudnick, 1997).

MDMA is transported into cells, in a similar manner to 5-HT, with sodium (Na^+) and chloride (Cl^-) ions in exchange for potassium ions (K^+). Intracellular deprotonation of MDMA enables outward diffusion of the neutral form, which is then reprotonated and becomes a substrate for SERT. This cycle may result in disruption of ionic gradients (Na^+, Cl^- and K^+), causing intracellular acidification.

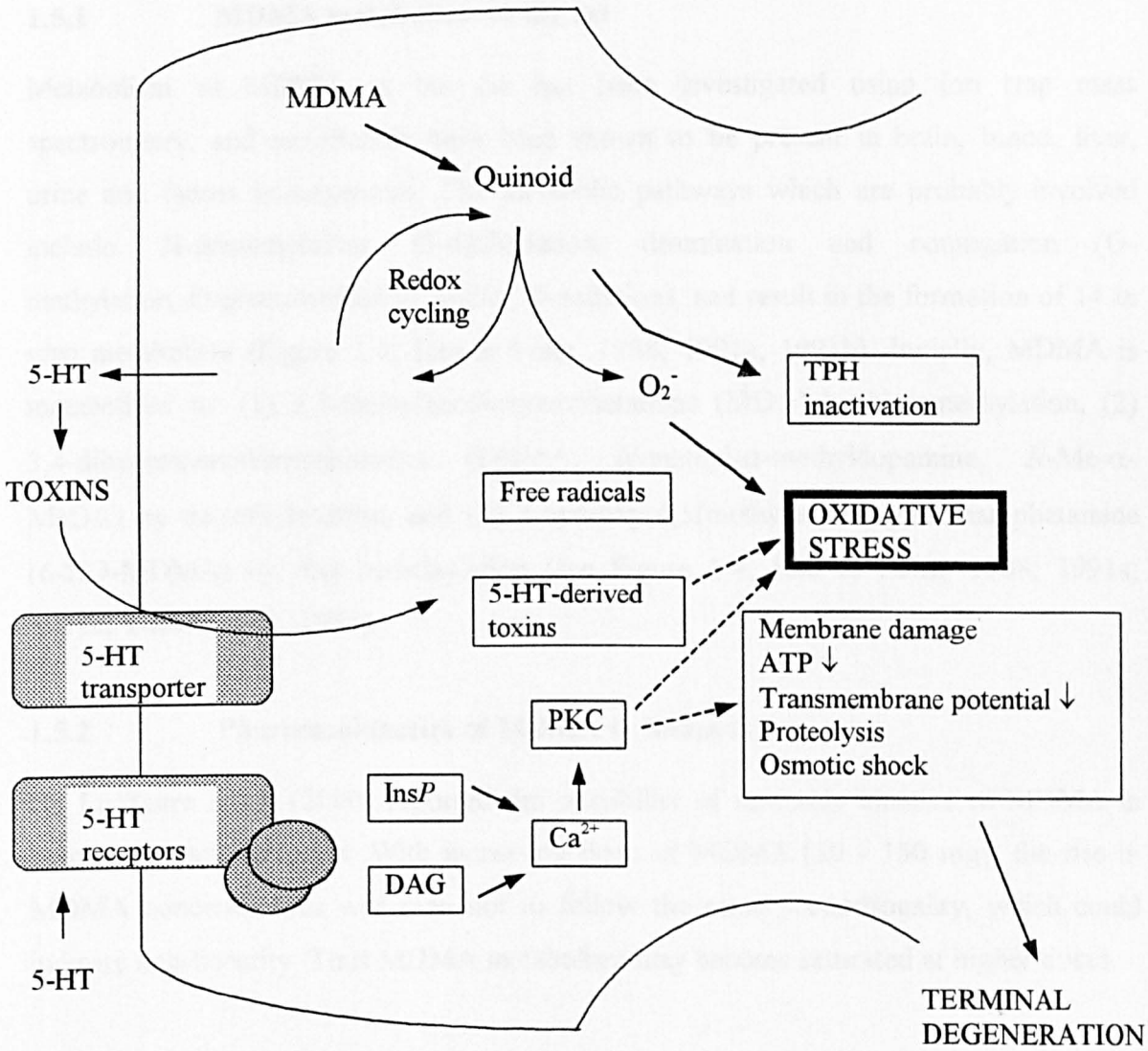

Figure 1.3 Postulated mechanisms of MDMA-induced toxicity (after Rattray, 1991).

MDMA could induce toxicity by: (1) metabolism to a quinoid, (2) generation of free radicals, (3) production of a 5-HT derived toxin, or (4) increasing intracellular calcium ion concentration. Neurotoxicity is likely to involve oxidative stress within the nerve terminal, which could lead to irreversible terminal degeneration.

Ca^{2+}: calcium ions; PKC: protein kinase C; Ins*P*: inositol triphosphate; DAG: diacylglycerol; TPH: tryptophan hydroxylase.

1.5 METABOLISM OF MDMA

1.5.1 MDMA metabolism in the rat

Metabolism of MDMA in the rat has been investigated using ion trap mass spectrometry, and metabolites have been shown to be present in brain, blood, liver, urine and faeces homogenates. The metabolic pathways which are probably involved include: N-demethylation, O-dealkylation, deamination and conjugation (O-methylation, O-glucoronidation, and/or O-sulfation), and result in the formation of 14 *in vivo* metabolites (Figure 1.4; Lim & Foltz, 1988; 1991a; 1991b). Initially, MDMA is metabolised to: (1) 3,4-methylenedioxyamphetamine (MDA) by N-demethylation, (2) 3,4-dihydroxymethamphetamine (DHMA; *N*-methyl-α-methyldopamine, *N*-Me-α-MeDA) by demethylenation, and (3) 2-hydroxy-4,5(methylenedioxy)methamphetamine (6-HO-MDMA) by ring hydroxylation (see Figure 1.4; Lim & Foltz, 1988; 1991a; 1991b; Tucker *et al.*, 1994).

1.5.2 Pharmacokinetics of MDMA in humans

De La Torre *et al.* (2000) reported the possibility of saturable kinetics of MDMA in human recreational users. With increasing dose of MDMA (50 - 150 mg), the rise in MDMA concentrations was seen not to follow the same proportionality, which could indicate non-linearity. Thus MDMA metabolism may become saturated at higher doses.

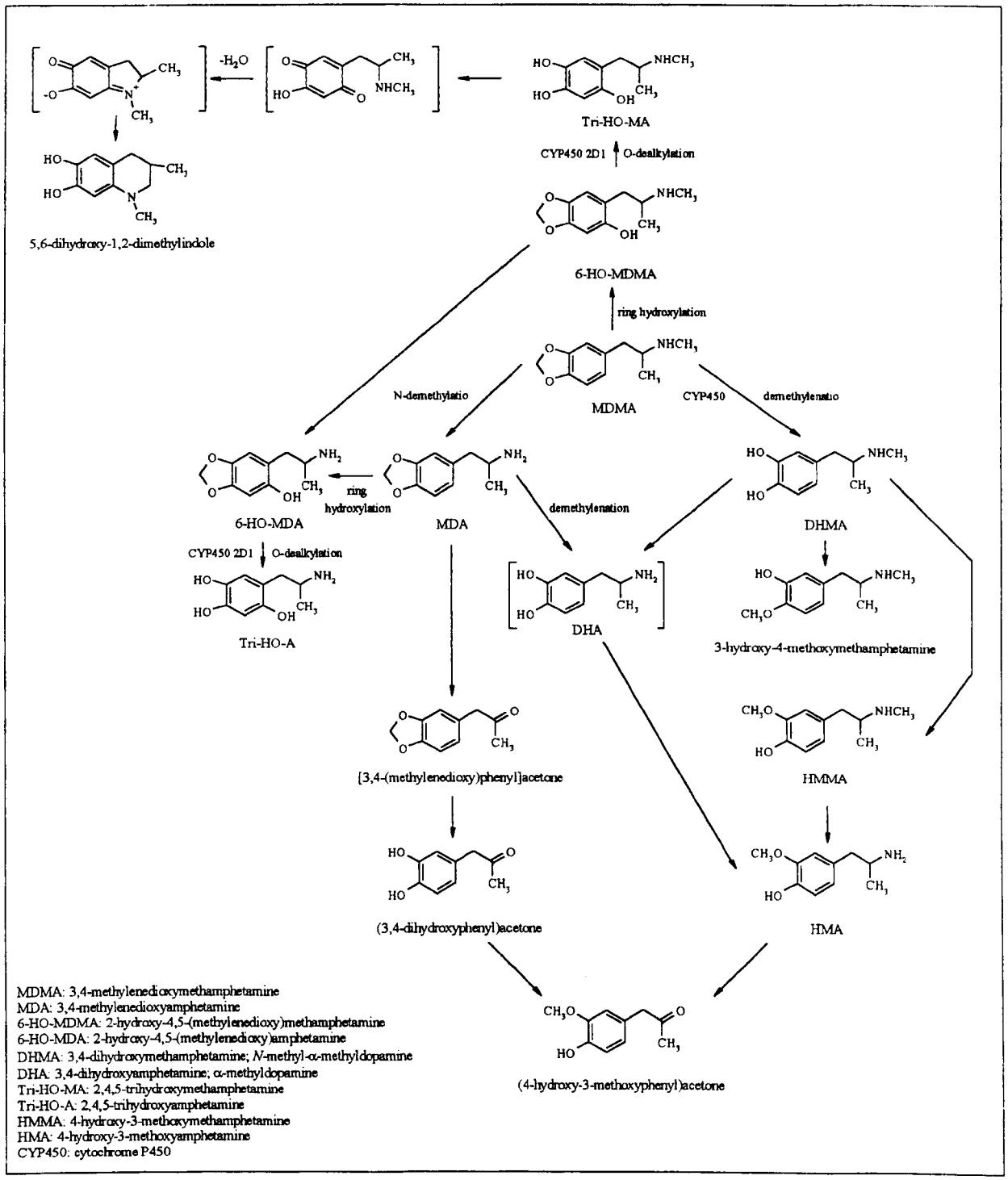

Figure 1.4 Postulated pathways of MDMA metabolism (after Hiramatsu *et al.*, 1990; Lim & Foltz, 1988; Lim & Foltz, 1991; Tucker *et al.*, 1994; Zhao *et al.*, 1992).

Structures in brackets are postulated intermediate compounds in the formation of 4-hydroxy-3-methoxyamphetamine and 5,6-dihydroxy-1,2-dimethylindole.

1.6 THE NEUROCHEMISTRY OF SEROTONIN

Serotonin is synthesised from the aromatic amino acid, L-tryptophan, which is hydroxylated to 5-hydroxy-L-trytophan (5-HTP) by tryptophan hydroxylase (TPH). 5-HTP is a short-lived intermediate product, being decarboxylated almost immediately by the non-specific enzyme, L-aromatic acid decarboxylase, in the cytoplasm to form 5-HT (see Figure 1.5). TPH is a specific enzyme found only in serotonin-synthesising cells and forms the rate-limiting step of serotonin synthesis. L-tryptophan is transported in plasma either in solution or bound to plasma proteins and is actively taken up into 5-HT nerve endings via an amino acid carrier mechanism. The amount of free L-tryptophan available in the presynaptic nerve terminal thus determines TPH activity and, ultimately, the rate of serotonin synthesis. Serotonin accumulates within storage granules, thought to be bound in a complex with proteins, divalent ions and adenosine triphosphate (ATP), and is released into the synaptic cleft following depolarisation of the axon terminal. Once released, uptake carriers present on the presynaptic nerve terminal remove serotonin from the synaptic cleft, transporting it back into the presynaptic neurone. Any serotonin which is not bound in storage vesicles, either before release or after reuptake, is metabolised to an aldehyde intermediate by monoamine oxidase (MAO), then oxidised to 5-HIAA by aldehyde dehydrogenase (see Figure 1.5; Fuller, 1985; Kruk & Pycock, 1991; Rang *et al.*, 1995).

There are currently 14 known mammalian 5-HT receptor subtypes (see Figure 1.6) which are, with the exception of the 5-HT_3 receptor (a ligand-gated ion channel), seven putative transmembrane spanning, G-protein coupled metabotropic receptors. Receptor localisation, agonists and antagonists for each subtype are displayed in Table 1.1. The behavioural and physiological responses mediated by serotonin include: temperature regulation, feeding behaviour, sexual behaviour, sleep and wakefulness, responding to painful stimuli, neuroendocrine responses, escape and stress, aggression, depression and anxiety (see Barnes & Sharp, 1999; Lucki, 1998; Roth *et al.*, 1998; Uphouse, 1997).

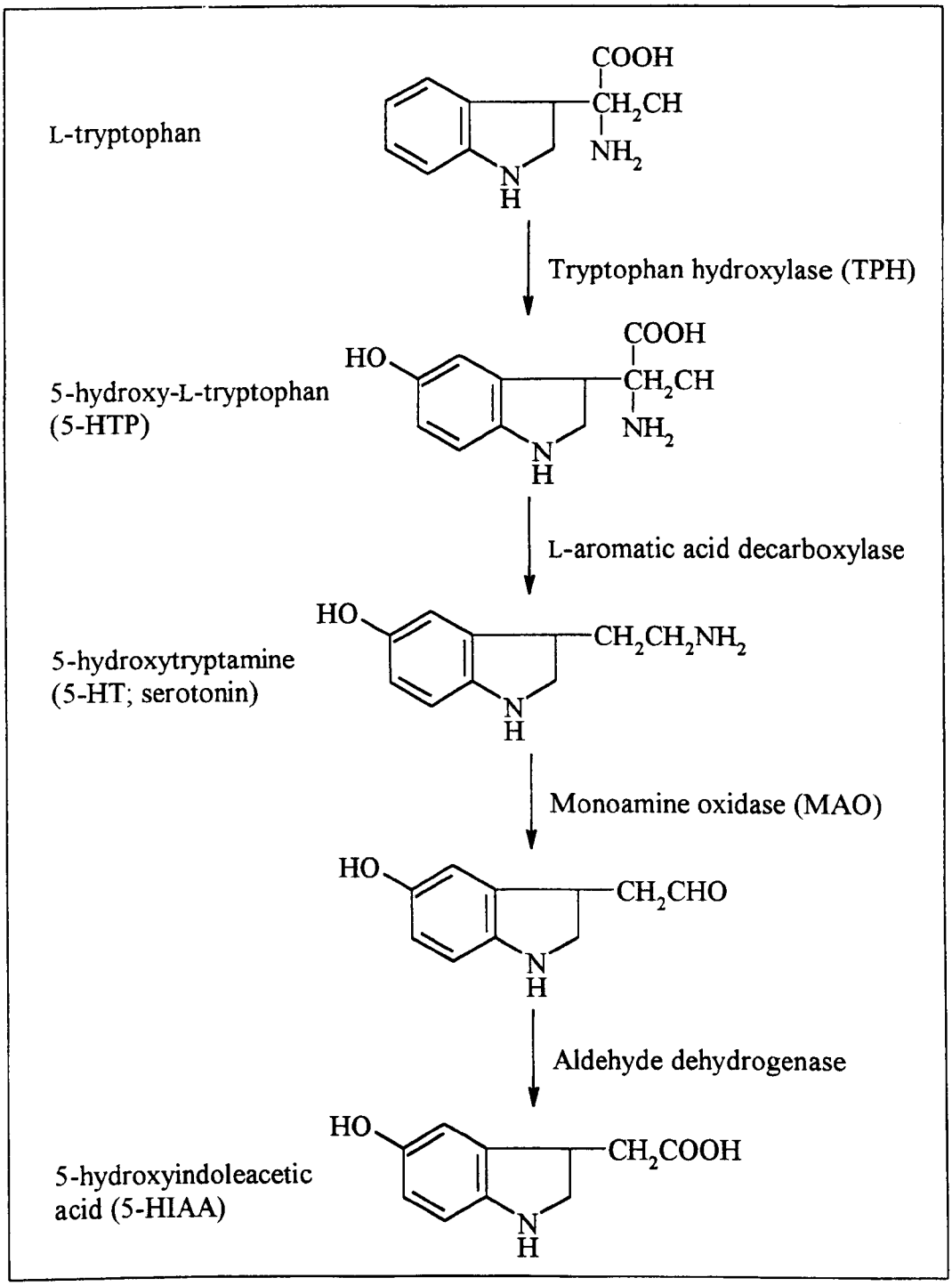

Figure 1.5 Synthesis and metabolism of 5-HT (see Fuller, 1985; Kruk & Pycock, 1991; Rang *et al.*, 1995).

L-tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH). 5-HTP is then decarboxylated to 5-hydroxytrytamine (5-HT) by L-aromatic acid decarboxylase. 5-HT is metabolised to 5-hydroxyindoleacetic acid (5-HIAA), via an aldehyde intermediate, by monoamine oxidase (MAO) and aldehyde dehydrogenase.

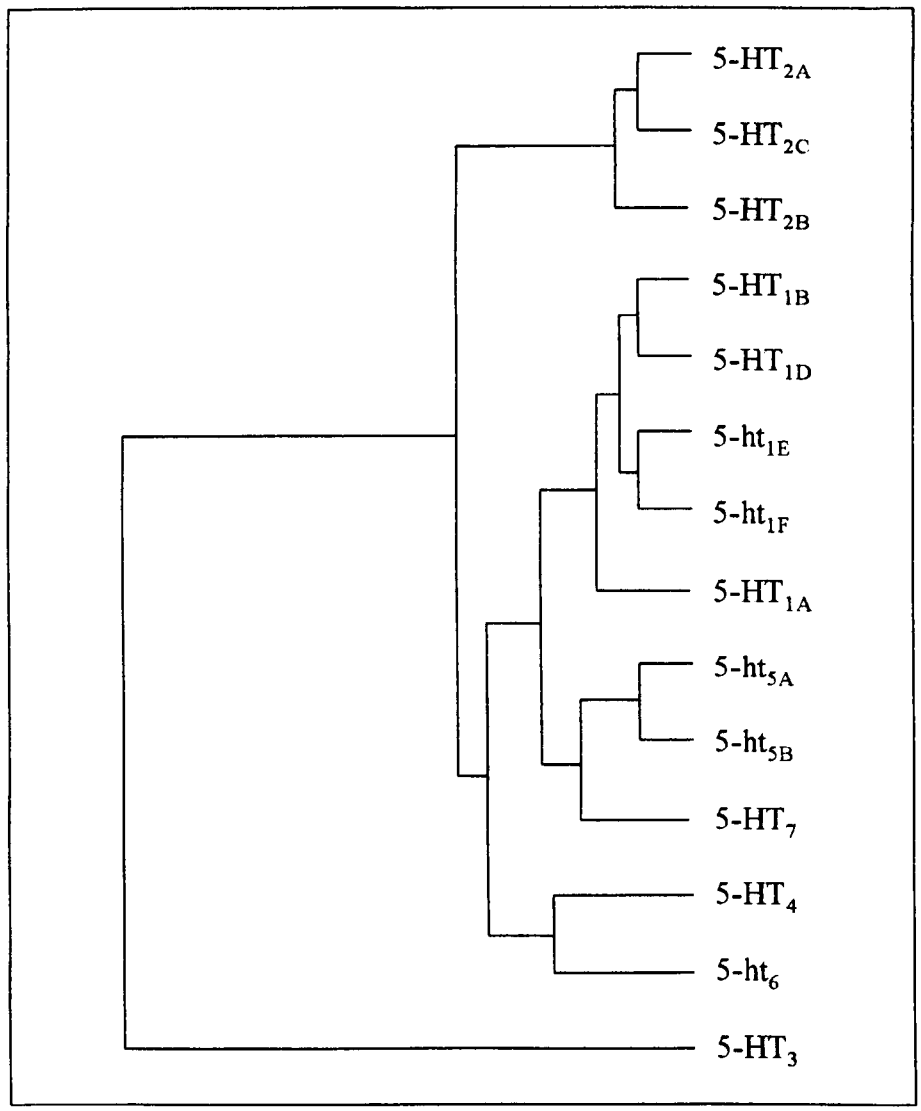

Figure 1.6 Dendrogram showing 5-HT receptor protein sequences (after Barnes & Sharp, 1999; Hoyer *et al.*, 1994).

Evolutionary relationship between human 5-HT receptor protein sequences (except 5-ht_{5A} and 5-ht_{5B} which are murine in origin). 5-HT receptors cluster into subgroups according to amino acid sequence similarity.

Receptor subtype	Location	Location within CNS	Response	Agonists	Antagonists
5-HT_{1A}	Neuronal, mainly CNS	Dorsal raphe nucleus, hippocampus, cortex	Neuronal hyperpolarisation, hypotension	8-OH-DPAT, buspirone, 5-CT	WAY 100635, WAY 100135, [methiothepin (non-selective)]
5-HT_{1B}	CNS and some peripheral nerves	Substantia nigra, basal ganglia, subiculum	Inhibition of neurotransmitter release	CP 93,129, 5-CT	SDZ 21009, [methiothepin (non-selective)]
5-HT_{1D}	Mainly CNS	Substantia nigra, basal ganglia, superior colliculus	Inhibition of neurotransmitter release	Sumatriptan, L 694247, 5-CT	GR 127935, [metergoline, methiothepin (non-selective)]
5-ht_{1E}	Only CNS	Cortex, caudate putamen, claustrum	Inhibition of adenylyl cyclase	5-HT	None [methiothepin - weak]
5-ht_{1F}	Mainly CNS	Cortex, hippocampus, claustrum, caudate nucleus	Inhibition of adenylyl cyclase	5-HT; sumatriptan	None [methiothepin - weak]
5-HT_{2A}	Vascular smooth muscle, platelets, lung, CNS, GI tract	Claustrum, olfactory tubercle, cortex, hippocampus, caudate nucleus, nucleus accumbens	Vasoconstriction, platelet aggregation, bronchoconstriction	α-methyl-5-HT, DOI	Ketanserin, cinanserin, ritanserin, MDL 100,907, pirenpirone
5-HT_{2B}	Mainly peripheral		Rat stomach fundic muscle contraction	α-methyl-5-HT, DOI	SB 200646, SB 242084
5-HT_{2C}	CNS - high density in choroid plexus	Choroid plexus, cortex, globus pallidus, substantia nigra, caudate nucleus, nucleus accumbens, hippocampus, amygdala	↑ Phosphoinositide turnover	α-methyl-5-HT, DOI	Mesulergine

Continued overleaf.

Receptor subtype	Location	Location within CNS	Response	Agonists	Antagonists
5-HT_3	Peripheral and central neurones	Dorsal vagal nerve, solitary tract nerve, trigeminal nerve, area postrema, spinal cord, hippocampus, amygdala	Depolarisation	2-methyl-5-HT, *m*-chlorophenyl-biguanide	Ondansetron, tropisetron, granisetron
5-HT_4	GI tract, CNS, heart, urinary bladder	Colliculus, hippocampus, nigrostriatal and mesolimbic systems	Activation of ACh release in gut, tachycardia, ↑ cAMP in CNS neurones	Metoclopramide, renzapride	GR 113808, SB 204070, tropisetron (weak)
5-ht_{5A} and 5-ht_{5B}	CNS		Not known	5-HT, 5-CT, LSD	Methiothepin
5-ht_6	CNS		Activation of adenylyl cyclase (HEK 293 cells)	5-HT	Methiothepin
5-HT_7	CNS		Activation of adenylyl cyclase (HeLa cells and COS cells)	5-HT	Methiothepin

Table 1.1 Operational characteristics of mammalian 5-HT receptors (after Barnes & Sharp, 1999; Hoyer *et al.*, 1994; Roth *et al.*, 1998; Uphouse, 1997).

CNS: central nervous system; ***GI tract***: gastrointestinal tract; ***ACh***: acetylcholine; ***cAMP***: cyclic adenosine monophosphate; ***8-OH-DPAT***: 8-hydroxy-2-(di-*n*-propylamino)tetralin; ***5-CT***: 5-carboxamidotryptamine; ***WAY 100635***: *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide; ***WAY 100135***: N-tert-butyl-3-[4-(2-methoxyphenyl) piperazin-1-yl]-2-phenylpropanamide; ***CP 93,129***: 3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one; ***SDZ 21009***: 4(3-terbutylamino-2-hydroxypropoxy)indol-2-carbonic acid-isopropylester; ***L 694247***: 2-[5-[3-(4-methylsulphonylamino)benzyl-1 2,4-oxadiazol-5-yl]-1H-indol-3-yl] ethanamine; ***GR 127935***: 2'-methyl-4'-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide; ***DOI***: (±)-2,5-dimethoxy-4-iodoamphetamine; ***MDL 100,907***: R-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol; ***SB 200646***: N-(1-methyl-5-indolyl)-N'-(3-pyridyl) urea; ***SB 242084***: 6-chloro-5-methyl-methyl-1-[2-(-methylpyridyl-3-oxy)-pyrid-5-yl carbamoyl]indoline; ***GR 113808***: 1-[2-(methylsulphonylamino)ethyl]4-piperidinyl]methyl-1-methyl-indole-3 carboxylate; ***SB 204070***: 8-amino-7-chloro-(N-butyl-4-piperidyl)methylbenzo-1,4-dioxan-5-carboxylate; ***LSD***: lysergic acid diethylamide; ***HEK 293***: human embryonic kidney 293 cells; ***HeLa***: human epithelial cells (cervical cancer); ***COS***: transformed monkey kidney fibroblast cells.

1.7 THE NEUROCHEMISTRY OF DOPAMINE

Dopamine is synthesised from the amino acid, L-tyrosine, which is hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH), this forming the rate-limiting step. TH is a relatively selective enzyme, being found only in catecholamine-containing cells (likely to be free in the cytosol). L-DOPA is decarboxylated by L-aromatic acid decarboxylase (DOPA decarboxylase), in the cytoplasm, to form dopamine (see Figure 1.7). Under normal conditions TH is completely saturated with L-tyrosine, therefore increased circulating L-tyrosine levels do not increase the rate of dopamine synthesis. TH activity is inhibited by the presence of high intraneuronal dopamine concentrations (end-product inhibition), leading to a decrease in further dopamine synthesis. L-DOPA is actively taken up into dopamine neurones, where it is converted to dopamine by DOPA decarboxylase. Dopamine accumulates in storage granules, as a complex with chromogranins, divalent metal ions and ATP. Dopamine is released into the synaptic cleft, via calcium-dependent exocytosis, following depolarisation of the axon terminal. Dopamine reuptake occurs following its release into the synaptic cleft, via a high-affinity, energy-dependent, active transport system. Following reuptake, any dopamine which is not bound in storage vesicles is metabolised by MAO and catechol-O-methyl transferase (COMT) to form 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA; 3-methoxy-4-hydroxyphenylacetic acid) (see Figure 1.7; Kruk & Pycock, 1991; Rang *et al.*, 1995).

There are currently two main classes of dopamine receptors: "D_1-like", comprising D_1 and D_5 receptor subtypes, and "D_2-like", comprising D_2, D_3 and D_4 receptor subtypes. Receptor localisation, agonists and antagonists for each receptor subtype are displayed in Table 1.2 (see Kruk & Pycock, 1991; Rang *et al.*, 1995; Strange, 2000). Dopamine is involved in the mediation of motor control, mood and emotion and neuroendocrine function (see Rang *et al.*, 1995).

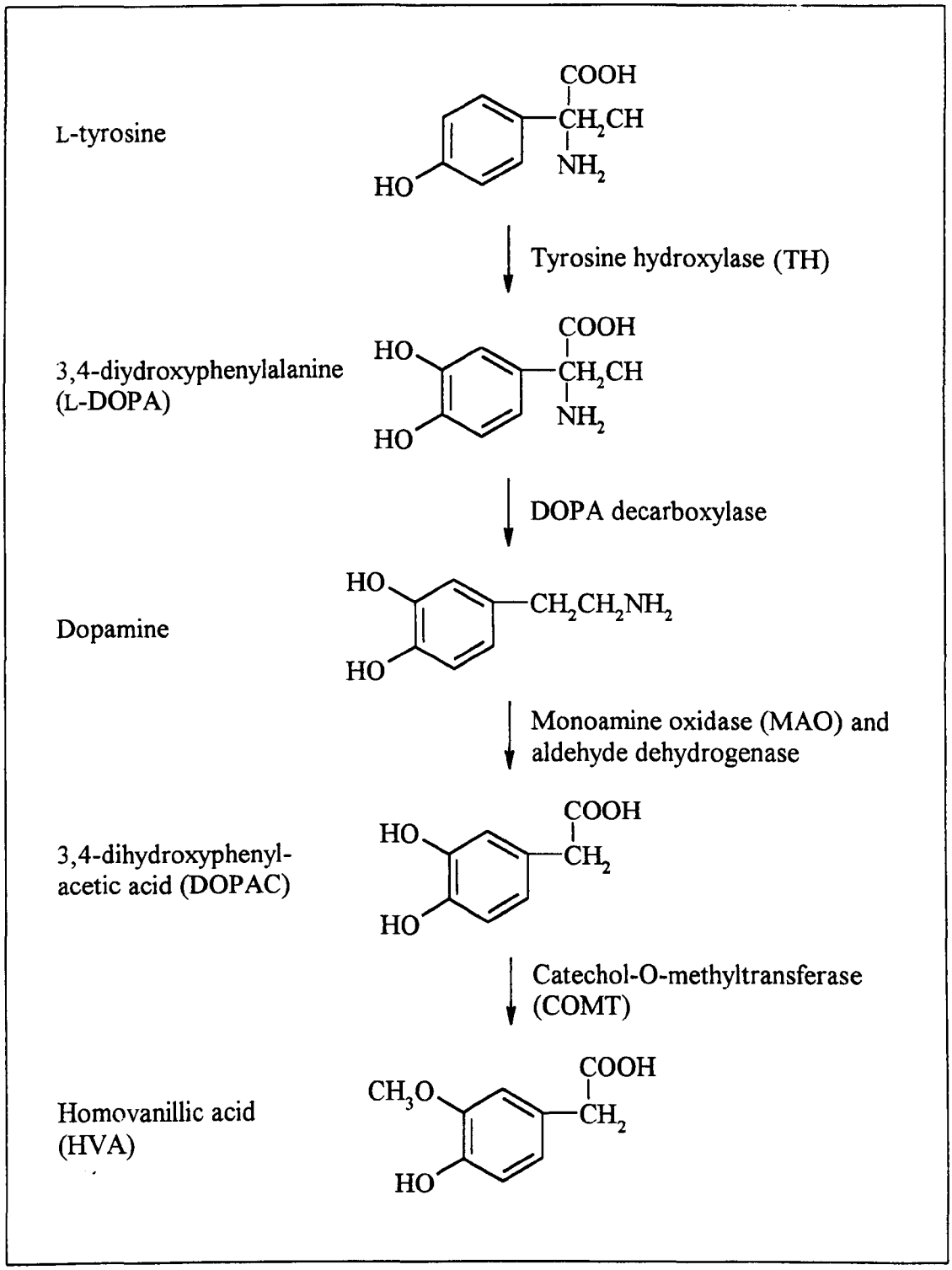

Figure 1.7 Synthesis and metabolism of dopamine (see Kruk & Pycock, 1991; Rang *et al.*, 1995).

L-tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH). L-DOPA is then decarboxylated to dopamine by DOPA decarboxylase. Dopamine is metabolised to 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO), then DOPAC is further metabolised to form homovanillic acid (HVA) by the action of catechol-O-methyltransferase (COMT).

Receptor subtype		Location within CNS	Response	Agonists	Antagonists
D_1-like	D_1	Caudate putamen, nucleus accumbens, olfactory tubercle, hypothalamus, thalamus, frontal cortex	↑ adenylyl cyclase, ↑ phospholipase C	SKF 38393 and dihydrexidine (selective agonists), dopamine (low potency), apomorphine and bromocriptine (partial agonists)	SCH 23390 and SKF 83566 (selective antagonists) phenothiazines and butyrophenones (low potency), clozapine (medium potency)
	D_5	Hippocampus, thalamus, striatum and cortex (all low densities)			
D_2-like	D_2	Caudate putamen, nucleus accumbens, olfactory tubercle, [cortex (low density)]	↓ adenylyl cyclase, ↑ K^+ channel, ↓Ca^{2+} channel	Quinpirole and N-0437 (selective agonists), dopamine, apomorphine and bromocriptine (high potency)	Sulpiride and nemonapride (selective antagonists), phenothiazines and spiperone (high potency, non-selective), butyrophenones (high potency, D_2-selective), clozapine (high potency, D_4-selective)
	D_3	Nucleus accumbens olfactory tubercle, [cortex (low density)]			
	D_4	Frontal cortex, midbrain, amygdala, hippocampus, hypothalamus, medulla (all low densities)			

Table 1.2 Operational characteristics of mammalian dopamine receptors (see Kruk & Pycock, 1991; Rang *et al.*, 1995; Strange, 2000).

Low potency: activity in the micromolar range; ***high potency***: activity in the nanomolar range; ***Phenothiazines***: chlorpromazine, thioridazine, fluphenazine (no selectivity between D_1 and D_2 receptors); ***Butyrophenones***: haloperidol, spiroperidol (some selectivity for D_2 receptors).

1.8 AIMS OF THE INVESTIGATION

The initial aims of this study were to investigate the longer-term functional consequences of MDMA administration in rats and to study the long-term neurotoxic effects in mice. There is now some reported evidence of long-term serotonergic deficits in humans (Kish *et al.*, 2000; McCann *et al.*, 1998, Semple *et al.*, 1999), in addition to evidence of lower impulsivity (McCann *et al.*, 1994) and altered behavioural responses to *m*-CPP (McCann *et al.*, 1999). Such data prompted the question as to whether MDMA administration has long-term functional consequences on behaviour in animals. Thus the behavioural effects of a single neurotoxic dose of MDMA (12.5 mg/kg i.p.), administered to Dark Agouti rats, were investigated at three time-points up to 80 days post-treatment using three models of anxiety-related and locomotor behaviour (elevated plus-maze, open field and automated activity meter).

Several studies have indicated a long-term effect of MDMA administration on thermoregulatory responses (Dafters & Lynch, 1998; Shankaran & Gudelsky, 1999) and 5-HT receptor activity (Aguirre *et al.*, 1998; McNamara *et al.*, 1995). The reported findings were further investigated by assessing: (1) the effects of three doses of MDMA, two or three weeks apart, on MDMA-induced acute hyperthermia, (2) the effects of a single dose of MDMA, five or six weeks earlier, on thermoregulatory responses under high (30 ± 0.5 °C) or low (10 ± 0.5 °C) ambient temperature conditions, and (3) the effects of a single dose of MDMA, three or four weeks earlier, on the hypothermic response to 8-OH-DPAT. The results obtained gave rise to an investigation of the mechanisms involved in the acute hyperthermic response. A series of drugs which affect serotonergic or dopaminergic function were administered in an attempt to determine which receptor subtype(s) might be involved in mediation of this response.

Finally, while MDMA administration to rats, guinea-pigs and non-human primates has been demonstrated to result in long-term serotonergic neurodegeneration, mice exhibit long-term depletion of dopamine and its metabolites (see Logan *et al.*, 1988; Stone *et al.*, 1987). Thus, a series of putative neuroprotective compounds were administered

prior to MDMA in an attempt to elucidate the mechanisms involved in its neurotoxic actions in this species.

All behavioural and thermoregulation experiments were conducted in Dark Agouti rats, which were chosen because a single dose of MDMA (10 - 15 mg/kg i.p.) produces a definable, reproducible, neurotoxic degeneration of 5-HT nerve endings in the brain, demonstrated by a 30 - 60 % reduction in regional 5-HT and 5-HIAA concentrations measured seven days post-injection (Colado *et al.* 1995; O'Shea *et al.* 1998). This is in contrast to other rat strains where several doses, or a single higher dose, of MDMA are required to produce significant damage to brain 5-HT nerve endings.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2 GENERAL MATERIALS AND METHODS

Details of specific methods used in this thesis are described in individual chapters. General materials and methods, relating to several chapters, are reported below.

2.1 ANIMALS

(1) De Montfort University

Adult male Dark Agouti (DA) rats, weighing 175 – 200 g at the start of each experiment, and adult male "Swiss"-type Albino (NIH/S) mice, weighing 25 – 30 g at the start of each experiment, were obtained from Harlan UK Ltd. (Bicester, Oxon.). Rats were housed in groups of three or four and mice in groups of between five and seven, in an ambient temperature of 20 ± 2 °C and a 12 h light/dark cycle (lights on: 07:30 h). Food (B & K Universal Ltd., Aldbrough, Hull) and water were provided *ad libitum*.

(2) University of Leicester

Adult male DA and Sprague-Dawley (SD) rats, weighing 200 – 260 g at the start of each experiment (Harlan UK Ltd.) were housed in groups of two or three, in an ambient temperature of 21 ± 2 °C and a 12 h light/dark cycle (lights on: 07:00 h). Food (Special Diet Services, Witham, Essex) and water were freely provided.

(3) Universidad Complutense

Adult male DA rats, weighing 150 - 175 g at the start of each experiment, and adult male Swiss-Webster mice (CFW1), weighing 30 – 35 g at the start of each experiment, were obtained from Interfauna (Barcelona, Spain). Rats were housed in groups of five and mice in groups of ten, in an ambient temperature of 21 ± 2 °C and a 12 h light/dark cycle (lights on: 07:00 h). Food and water were freely provided.

All experiments were performed according to guidelines set by De Montfort University, the University of Leicester and Universidad Complutense concerning the use of

experimental animals and under appropriate project and personal licence authority granted under the United Kingdom Animals (Scientific Procedures) Act, 1986.

2.2 DRUGS

Drugs were obtained from the following sources:

- Diazepam, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI), 1-[2-*bis* (4-fluorophenyl) methoxy]ethyl]-4-3-phenylpropyl]piperazine dihydrochloride (GBR 12909), 8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT), (±) 3,4-methylenedioxymethamphetamine hydrochloride (MDMA), 7-nitroindazole (7-NI) and α-phenyl-N-tert-butyl nitrone (PBN) (Sigma-Aldrich Company Ltd., Poole, Dorset). MDMA was also obtained from Dr P Guiry (University College, Dublin) and NIDA (Research Triangle Park, North Carolina, USA).
- 1-(3-chlorophenyl)piperazine (*m*-CPP), α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11,939), (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine; (+)-MK 801 maleate) and R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzapine (SCH 23390) (Tocris Cookson Ltd., Avonmouth, Bristol).
- S-methyl-L-thiocitrulline (S-MTC) (Calbiochem, San Diego, California, USA).
- S-(+)-α-phenyl-2-pyridine ethanamide dihydrochloride (AR-R15896AR) and N-(4-(2-((3-chlorophenylmethyl) amino)-ethyl)phenyl) 2-thiophene carboxamidine (AR-R17477AR) (gifts from AstraZeneca, R&D Wilmington, Delaware). Clomethiazole edisilate, (S(-))-remoxipride hydrochloride and (cis)-zimeldine (gifts from AstraZeneca, R&D Södertälje, Sweden).
- Ritanserin (gift from Janssen Research Foundation, Janssen Pharmaceutica N.V., Beerse, Belgium).
- Methysergide hydrogen maleate (gift from Novartis International AG, Basel, Switzerland).
- R-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol (MDL 100,907) and 6-chloro-5-methyl-methyl-1-[2-(-methylpyridyl-3-oxy)-pyrid-5-yl carbamoyl]indoline (SB 242084) (gifts from Dr G Kennett, Vernalis Ltd., Wokingham).

All drugs were dissolved in normal saline (0.9 % w/v), unless otherwise specified, and injected intraperitoneally in volumes of 1 ml/kg for rats and 10ml/kg for mice, unless otherwise stated. Doses given were calculated as the base weight, unless otherwise specified.

2.3 CHEMICALS

Chemicals were obtained from the following sources:

2.3.1 Vehicles for injection

- Sodium chloride (Fisher Scientific UK, Loughborough, Leicestershire) was dissolved in deionised water (USF Elga Ltd., High Wycombe, Buckinghamshire), at a concentration of 0.9 % w/v.
- Tween 20 (BDH Laboratory Supplies, Poole, Dorset).
- Glacial acetic acid (Fisher).
- Dimethylsulphoxide (DMSO) (Fisher).
- Citric acid (Sigma).
- β-cyclodextrin (Sigma).
- Polyethylene glycol (PEG) (Fisons, Ipswich, Suffolk).

2.3.2 Chemicals for h.p.l.c.

- Components of the mobile phase utilised for high performance liquid chromatography (h.p.l.c.) were specified for h.p.l.c. with electrochemical detection where possible, and included potassium dihydrogen orthophosphate (KH_2PO_4), 1-octane sulphonic acid sodium salt (OSA), diaminoethanetetra acetic acid disodium salt (EDTA), methanol and orthophosphoric acid (Fisher).
- Standards used for h.p.l.c. were dissolved in a 0.2 M perchloric acid solution containing 0.1 % w/v L-cysteine, 0.1 % w/v sodium metabisulphite and 0.01 % w/v EDTA (Fisher).
- Standards used were: 5-hydroxytryptamine creatinine sulphate complex (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), 3-hydroxytyramine (dopamine), 3,4-

dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxy-phenylacetic acid (homovanillic acid, HVA) (Sigma).

2.3.3 General chemicals

- Cider vinegar, used for cleaning of behavioural testing apparatus (Tesco, Cheshunt, Hertfordshire).

2.4 GENERAL EQUIPMENT

- Tissue dissection equipment (Richardsons of Leicester Ltd., Leicester).
- AG104 analytical balance (Mettler-Toledo Ltd., Beaumont Leys, Leicester).
- POLYTRON PT 3100 homogeniser (Kinematica, Luzern, Switzerland).
- MSE Soniprep 150 ultrasonic disintegrator (Sanyo Gallenkamp Plc., Loughborough, Leicestershire).
- Hettich Mikro Rapid/K centrifuge (Sartorius Ltd., Epsom, Surrey).
- Millipore solvent filtration system, with Durapore 45 µm membrane filters (Millipore (UK) Ltd, Watford, Hertfordshire).
- Vacuum pump (KNF Neuberger, Freiburg, Germany).
- Sonicating water bath (Ultrawave Ltd., Cardiff).
- RE357 Microprocessor pH meter (EDT Instruments Ltd., Dover, Kent)
- Accumet gel-filled pH electrode (Fisher).
- SM11 magnetic stirrer (Stuart Scientific, Bibby Sterilin Ltd., Stone, Staffordshire).
- Variable volume pipettors, 20 – 200 µl, 200 – 1000 µl and 1000 – 5000 µl (Fisher).

2.5 STATISTICS

Statistical analysis was performed using GraphPad Prism (San Diego, California, USA) and BMDP/386 Dynamic (BMDP Statistical Solutions Ltd., Cork, Eire). All data is presented as mean ± s.e.m.

2.6 BEHAVIOURAL TESTING

2.6.1 Elevated plus-maze apparatus and behavioural assessment

The elevated plus-maze (Figure 2.1; on loan from Professor C. Marsden, School of Biomedical Sciences, University of Nottingham) was constructed from black plastic and positioned 50 cm above the floor. The maze consisted of two open arms (each 16 x 46 cm), two closed arms (each 16 x 46 cm, with side and end walls of height 10 cm) and a central square (16 x 16 cm). Each arm was divided, by a white line, into two zones; Zone 1 being that closest to the central square and Zone 2 being that closest to the distal end of the arm. The apparatus was situated in a darkened room, illuminated by a single 60 W white light bulb positioned approximately 50 cm above the central square. Experiments were recorded by a video camera suspended approximately 100 cm above the centre of the plus-maze. A weak cider vinegar solution (10 %) was used to clean the apparatus prior to the introduction of each animal.

Rats were tested on the maze in randomised order. Each rat was placed in the centre of the plus-maze facing one of the open arms and observed for 10 min by an experimenter seated approximately 1 m from the apparatus for behavioural scoring purposes. Arm entries were counted as four paw entries into either closed or open arms, while the time spent in different arms was calculated by subsequent analysis of video-recordings. Total arm entry data enabled calculation of the percentage (%) number of open arm entries and the % time spent on open arms. A number of simple behaviours were also noted, including: rearing (sitting on the hind paws only, apart from whilst grooming), head dips (lowering the head, either over the edge of an open arm or over the surrounding wall of a closed arm), stretched attend postures (SAP; full body stretch, usually with hindpaws in the central zone and forepaws in an open arm zone), directed sniffing (sniffing of the base or surrounding wall of the maze), non-directed sniffing (sniffing the air, often whilst rearing with forepaws positioned on the top edge of the surrounding wall), grooming and defecation.

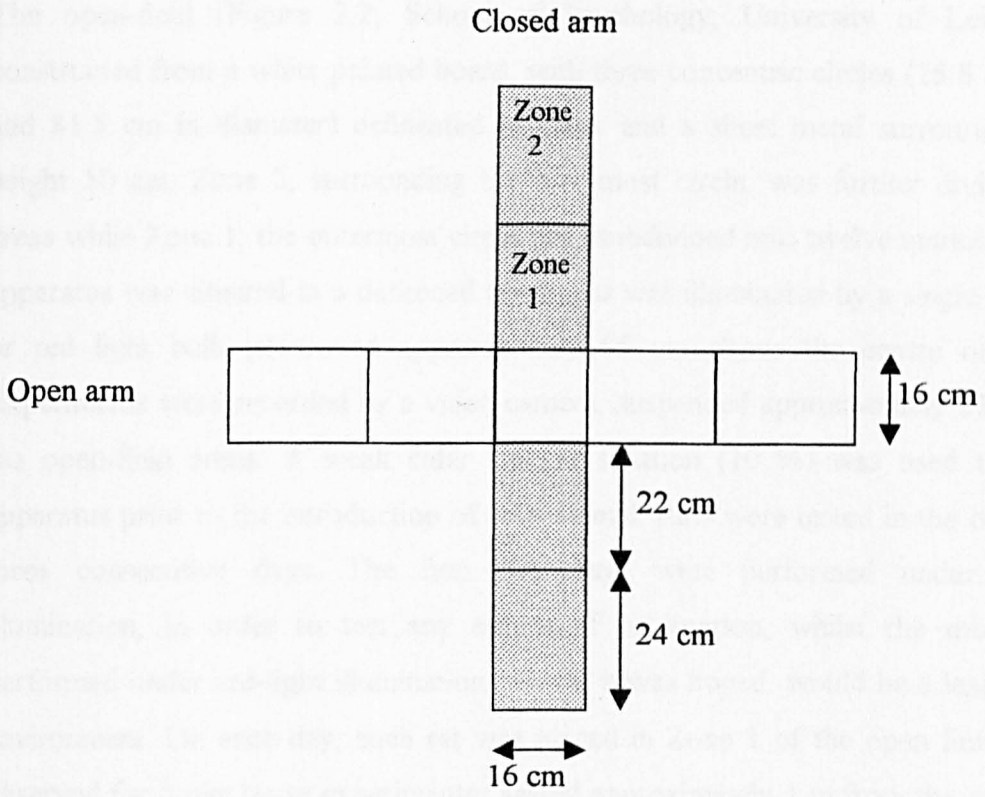

Figure 2.1 Plan diagram of the elevated plus-maze.

The plus-maze was constructed from black plastic and was elevated 50 cm above the floor. All arms were 16 cm wide and 46 cm long and each was divided into two sections: Zone 1 (proximal to the centre) and Zone 2 (distal from the centre). The closed arms had 10 cm high walls.

2.6.2 Open-field apparatus and behavioural assessment

The open-field (Figure 2.2; School of Psychology, University of Leicester) was constructed from a white painted board, with three concentric circles (15.5 cm, 45.5 cm and 81.5 cm in diameter) delineated in black and a sheet metal surrounding wall of height 50 cm. Zone 2, surrounding the innermost circle, was further divided into six areas while Zone 1, the outermost circle, was subdivided into twelve marked areas. The apparatus was situated in a darkened room and was illuminated by a single 60 W white or red light bulb positioned approximately 65 cm above the centre of the arena. Experiments were recorded by a video camera, suspended approximately 150 cm above the open-field arena. A weak cider vinegar solution (10 %) was used to clean the apparatus prior to the introduction of each animal. Rats were tested in the open-field on three consecutive days. The first two days were performed under white-light illumination, in order to test any effects of habituation, whilst the third day was performed under red-light illumination, which, it was hoped, would be a less anxiogenic environment. On each day, each rat was placed in Zone 1 of the open field arena and observed for 5 min by an experimenter seated approximately 1 m from the apparatus for behavioural scoring purposes. Locomotor behaviour was recorded by noting the total number of crossings within each zone with all four paws. The behaviours observed were: freezing (no movement within any particular section), rearing, grooming and defecation.

2.6.3 Automated activity meter apparatus and behavioural assessment

The automated activity meter (Figure 2.3; Benwick AM1051 Activity Monitor, Linton Instrumentation, Diss, Norfolk) was a metal framework (dimensions 55.5 cm x 33.5 cm x 17.5 cm) inside which a test cage was placed containing a single animal. The unit was enclosed in a wooden box, without any illumination. The apparatus used an array of 44 infrared beams, including a lower and upper set, enabling monitoring of horizontal and rearing activity. Data (numbers of lower and upper level beam breaks) was electronically logged at 5 min intervals for a total of 1 h. Four animals were tested at any particular time, in four separate activity monitor boxes. In each case, a rat was placed in a test cage immediately prior to the cage being placed in the activity monitor framework and the data logger switched on.

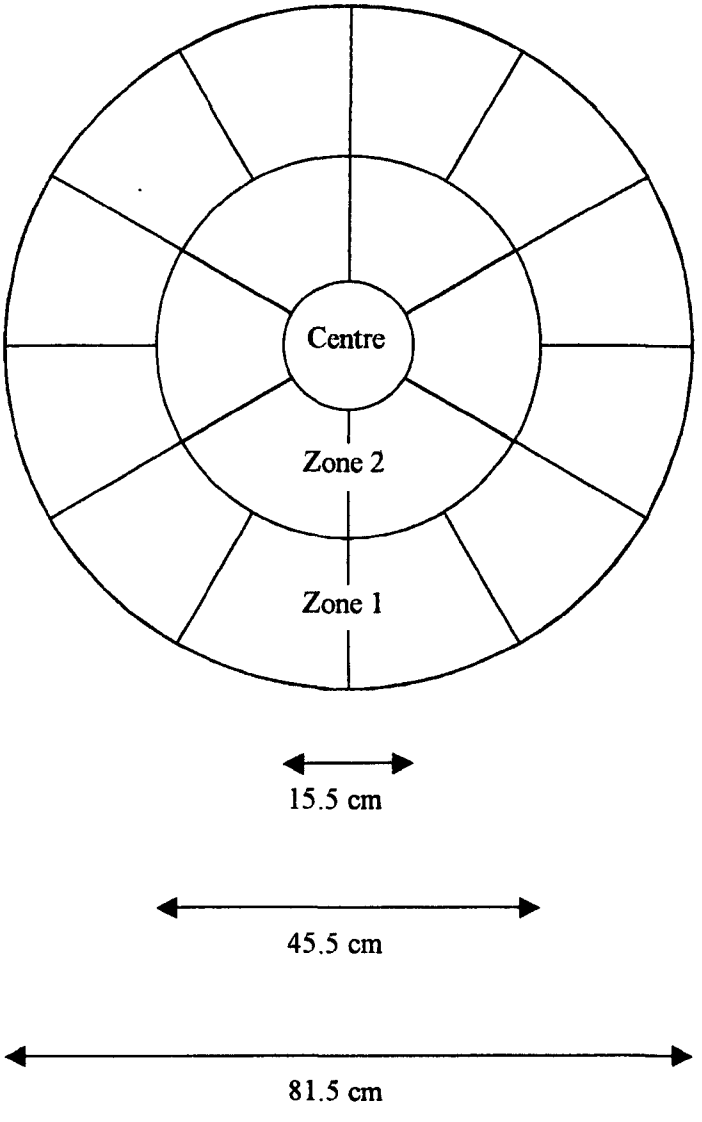

Figure 2.2 Plan diagram of the open-field arena.

The open-field comprised a white painted board, with three concentric circles delineated in black: Centre, 15.5 cm; Zone 2, 45.5 cm; Zone 1, 81.5 cm diameter. Zone 2 was divided into 6 sections and Zone 1 was divided into 12 sections. A 50 cm high sheet metal wall surrounded the outermost circle.

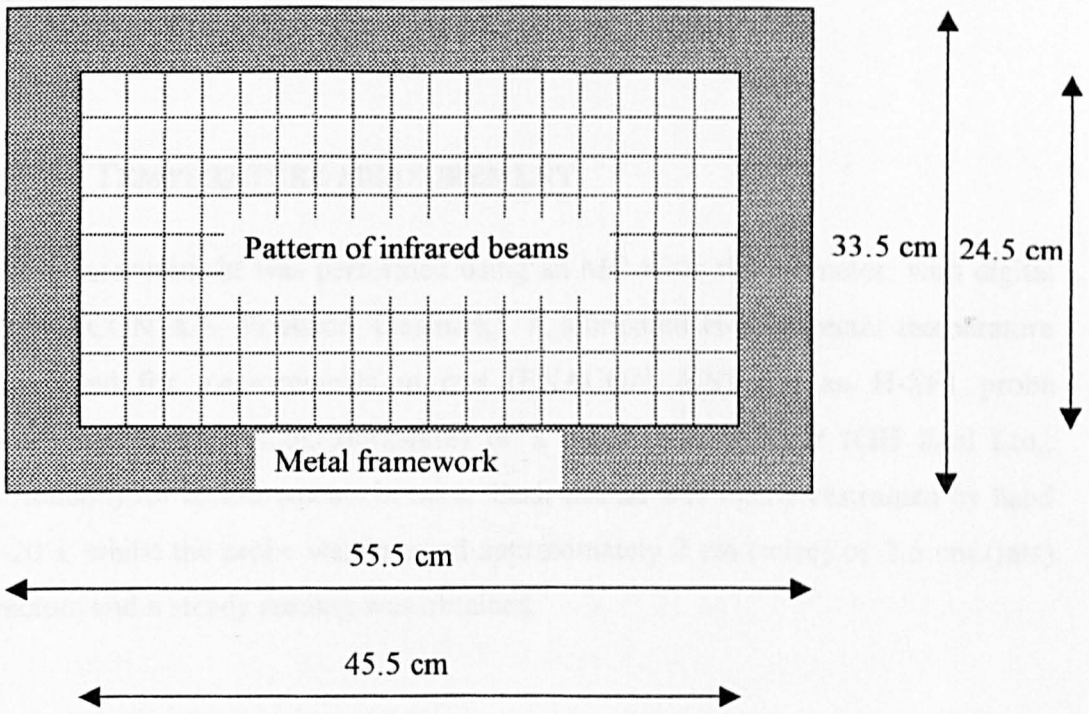

Figure 2.3 Plan diagram of the automated activity meter.

The automated activity meter comprised a metal framework, which was 55.5 cm long, 33.5 cm wide and 17.5 cm high (Benwick AM1051 Activity Monitor). A test cage containing a single animal was placed inside the framework and the array of infrared beams enabled monitoring of total and vertical (rearing) activity.

2.6.4 Video-monitoring equipment

- Black and white CCD pinhole board camera, 60 degrees field of view (Maplin Electronics Plc., Barnsley, South Yorkshire).
- 14 " combined TV/video (Goodmans TVC141, Comet, Hull).

2.7 TEMPERATURE MEASUREMENT

Temperature measurement was performed using an MC 8700 thermometer, with digital readout (EXACON A/S, Roskilde, Denmark). A lubricated H-RB3 rectal temperature probe was used for measurements in rats (EXACON A/S) and an H-SF1 probe (Cranbrook Electronics, Ascot, Berkshire) or a digital thermometer (GH Zeal Ltd., Merton, London) for measurements in mice. Each animal was lightly restrained by hand for 10 - 20 s, whilst the probe was inserted approximately 2 cm (mice) or 2.5 cm (rats) into its rectum and a steady reading was obtained.

2.8 BRAIN DISSECTION AND TISSUE PROCESSING

Rats and mice were sacrificed by cervical dislocation and decapitation, the brains rapidly removed and the cortex, hippocampus, striatum and hypothalamus dissected out on ice. Tissue was wrapped in labelled aluminium foil pieces and stored on ice until transferred to a -80 °C freezer prior to further processing. Prior to biochemical analysis, tissue was weighed and placed in plastic tubes containing 0.2 M perchloric acid solution (see section 2.3). The size of tube and volume of perchloric acid solution were varied depending on the size of the tissue: (1) for rat cortical and hippocampal tissue 13 ml polycarbonate tubes were used, containing 2 ml and 1 ml of liquid, respectively; (2) for rat striatal and hypothalamic tissue 6 ml polycarbonate tubes were used, containing 1 ml and 500 µl of liquid, respectively; (3) for mouse cortical tissue 6 ml polycarbonate tubes, containing 2 ml of liquid; and (4) for mouse hippocampal, striatal and hypothalamic tissue 1.5 ml microcentrifuge tubes were used, containing 300 µl, 500 µl and 250 µl of liquid, respectively. All rat brain tissue samples and mouse cortical tissue samples were subsequently homogenised (POLYTRON PT 3100) at 20,000 r.p.m. for 25 – 30 s. All remaining mouse brain tissue samples were homogenised by ultrasonic

disintegration (MSE Soniprep 150) at a rate of 23 kHz and an amplitude of 14 – 18 microns for 15- 20 s. All tissue homogenates were then centrifuged at 12 000 r.p.m. for 20 min at 4 °C, the supernatants decanted and stored at -80 °C, until analysed by h.p.l.c. with electrochemical detection (see section 2.9.6).

2.9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.9.1 Apparatus

The apparatus used for the biochemical analysis of regional brain amine concentrations included:

- An LKB 2150 HPLC pump (Amersham Pharmacia Biotech Limited, Little Chalfont, Buckinghamshire), a 7125-081 manual injector with a 50 µl loop (Rheodyne L.P., California, USA) and a Hamilton syringe for sample injection.
- A Shimadzu Liquid Chromatograph LC-9A pump (Shimadzu UK, Milton Keynes) and an automatic Jasco Intelligent Sampler 851-AS with a 100 µl injection loop programmed to deliver 50 µl samples (Jasco UK Ltd., Great Dunmow, Essex), and a Phenomenex Degassex DG-440 in-line degasser (Phenomenex, Macclesfield, Cheshire).
- A C^{18} Microsorb stainless steel reversed-phase column (150 x 4.6 mm), with an octadecyldimethylsilane monolayer bonded phase of particle size 5 µm and pore size 100 Å (Anachem Ltd., Luton, Bedfordshire).
- A C^{18} Lichrospher RP-Select B stainless steel reversed-phase column (150 x 4.6 mm), with a particle size of 5 µm (Phenomenex).
- An LC-4C amperometric detector (Bioanalytical Systems Inc. (BAS), Congleton, Cheshire), comprising a dual glassy carbon working electrode with an applied potential of 800 mV, versus a silver/silver chloride gel reference electrode.
- An SP4400 ChromJet integrator (ThermoQuest UK, Wythenshawe, Manchester).
- Nelson Analytical 900 Series Interface and 2600 Chromatography software, Version 5.1.5 (PE Nelson Systems Inc., LABWORKS, Baton Rouge, Louisiana, USA).

2.9.2 Amperometric detector calibration

Each standard was injected, in triplicate, on to the h.p.l.c. column over a range of applied potential of the working electrode, to ascertain the voltage at which optimum oxidation of each compound occurred (5-HT and 5-HIAA: 400 – 800 mV; dopamine: 400 – 950 mV; DOPAC: 400 – 900 mV; HVA: 600 mV – 1 V). Voltage was then plotted against peak height (Figure 2.4) and values within the plateau of each resulting curve were deemed optimum for future h.p.l.c. analysis, 800 mV being chosen.

2.9.3 Standard calibration curves

Each standard was injected, in triplicate, on to the h.p.l.c. column over a range of concentrations in order to ascertain suitable values for calculation of tissue levels of monoamines and their metabolites (5-HT and 5-HIAA: 5 – 40 ng/ml; dopamine: 50 – 400 ng/ml; DOPAC and HVA: 10 – 80 ng/ml). Concentration was then plotted against peak height (Figure 2.5) and demonstrated linear relationships in each case, enabling the choice of suitable concentrations for future h.p.l.c. analysis (see section 2.9.5).

2.9.4 Mobile phase

The mobile phase consisted of KH_2PO_4 (0.05 M), OSA (0.25 mM), EDTA (0.1 mM) and methanol (13 %) and was adjusted to pH 3.0 with orthophosphoric acid. The solution was vacuum-filtered and degassed via sonication and was subsequently pumped at a rate of 1.25 ml/min.

2.9.5 Standards

H.p.l.c. standards were initially dissolved at concentrations of 1 mg/ml in 0.2 M perchloric acid solution (see section 2.3). Following the development of calibration curves, the final concentrations of each standard injected on to the column were as follows: 5-HT and 5-HIAA: 25 ng/ml; dopamine: 250 ng/ml; DOPAC and HVA: 50 ng/ml.

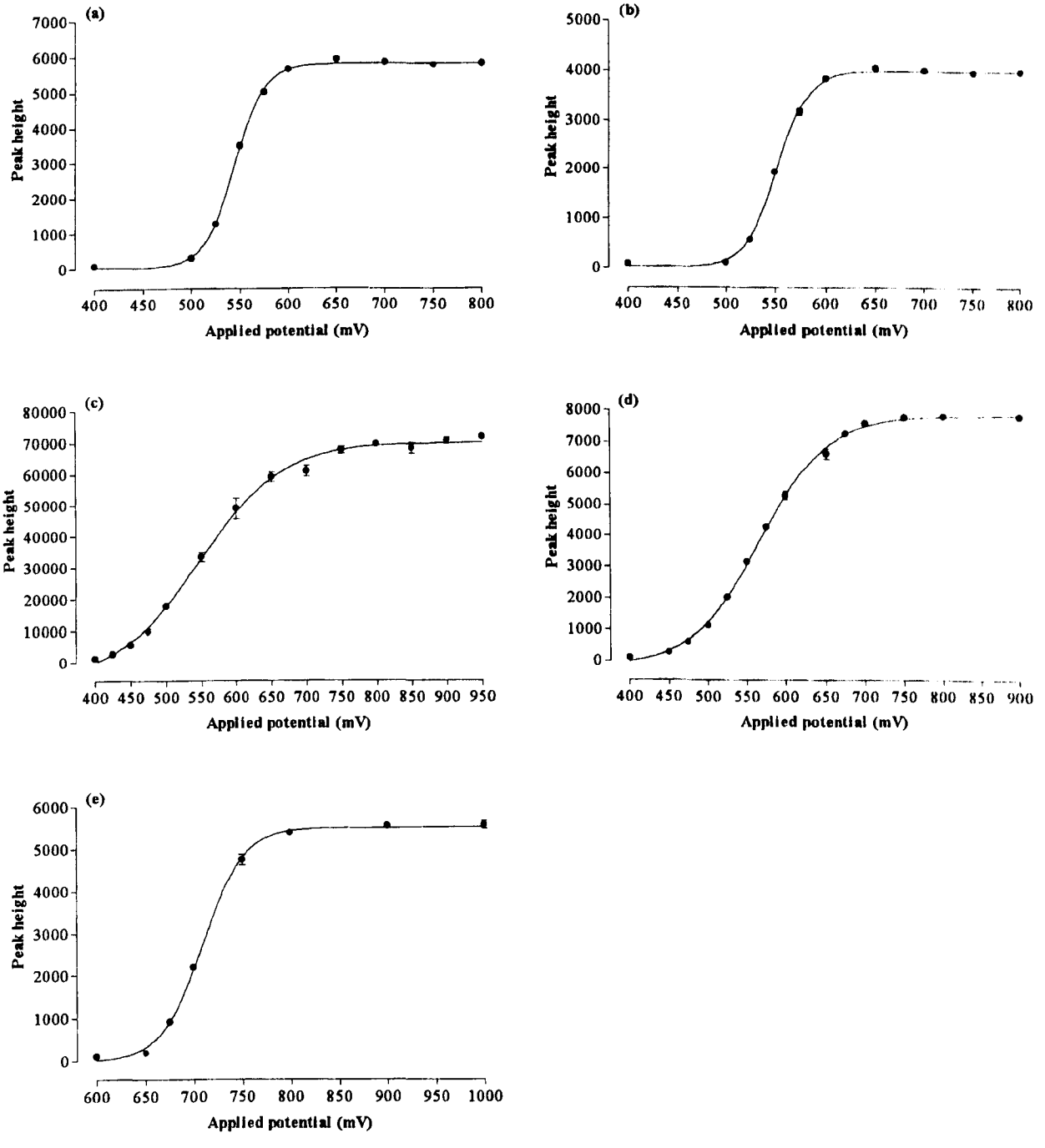

Figure 2.4 Measurement of the applied potential of the working electrode to ascertain the correct voltage for optimal oxidation of monoamines and their metabolites.

Results shown as mean ± s.e.m. (n = 3). ***(a)*** 5-HT, ***(b)*** 5-HIAA, ***(c)*** dopamine, ***(d)*** DOPAC, ***(e)*** HVA.

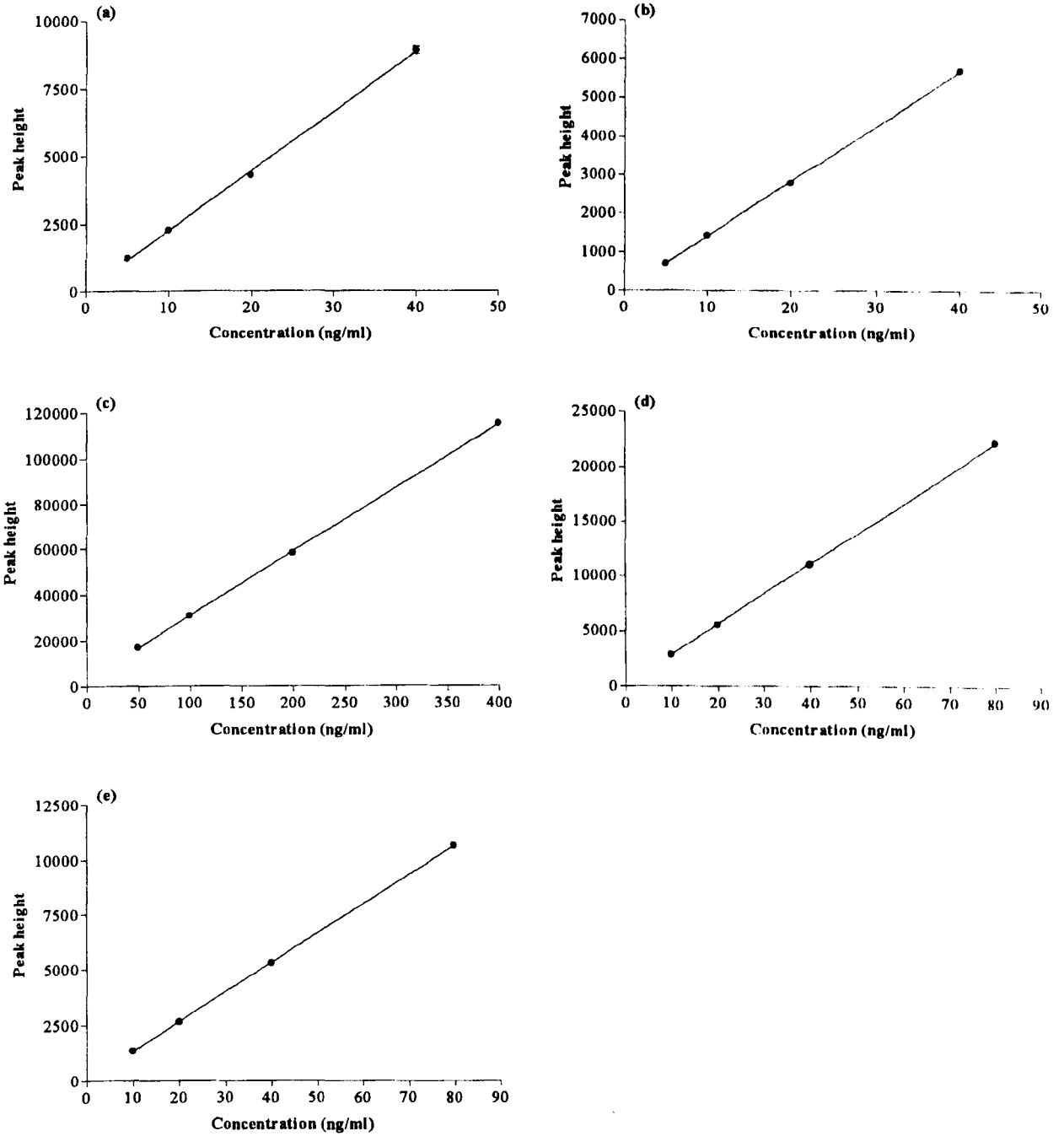

Figure 2.5 Standard calibration curves to determine suitable values for calculation of tissue levels of monoamines and their metabolites.

Results shown as mean ± s.e.m. (n = 3). ***(a)*** 5-HT, ***(b)*** 5-HIAA, ***(c)*** dopamine, ***(d)*** DOPAC, ***(e)*** HVA.

2.9.6 Measurement of 5-HT, dopamine and their metabolites in regional brain tissue

Following dissection and processing of regional brain tissue (see section 2.8), samples were analysed by h.p.l.c. with electrochemical detection. The mobile phase flow rate was set at 1.25 ml/min, the working electrode potential was 800 mV with a filter rate of 0.10 Hz and the detector range was set at 10 nA. In each experiment, the relevant standard solutions were injected on to the h.p.l.c. column at regular intervals, interspersed between injections of tissue sample supernatants. The integrator or computerised data collection system calculated peak height data, which thus enabled determination of levels of 5-HT, 5-HIAA, dopamine, DOPAC and HVA in tissue samples by comparing the peak heights of known standard solutions with those of samples:

1. $$\left(\frac{[\text{std}]\ (\text{ng/ml})}{\text{std PH}}\right) \times \text{ts PH} = [\text{ts}]\ (\text{ng/ml})$$

2. $$\frac{[\text{ts}]}{\text{ts W (g)}} = [\text{ts}]\ (\text{ng/g tissue})$$

(where [ ] = concentration; std = standard; ts = tissue; PH = peak height; W = weight).

CHAPTER 3

BEHAVIOURAL TESTING I:

A COMPARISON BETWEEN DARK AGOUTI AND SPRAGUE-DAWLEY RATS IN EXPERIMENTAL MODELS OF ANXIETY

3 BEHAVIOURAL TESTING I: A comparison between Dark Agouti and Sprague-Dawley rats in experimental models of anxiety

3.1 INTRODUCTION

3.1.1 Animal models of anxiety

Anxiety cannot directly be measured in animals; rather behavioural models measure adaptive responses to specific stimuli (see Handley *et* al., 1993). However, a variety of animal models of "anxiety" have been developed and are broadly grouped, according to the behaviours under investigation, into conditioned and unconditioned tests.

3.1.1.1 Conditioned tests

Conditioned "anxiety" tests include fear-potentiated startle and conflict tests, such as the Geller-Seifter and Vogel tests, and tend to require subject training followed by the presentation of some form of aversive stimulus (see Rodgers, 1997; Rodgers & Dalvi, 1997).

(1) Fear-potentiated startle

Startle reflexes are often characterised by a whole body jump in the rat (see Richardson, 2000) and may be investigated in the following manner: Brown *et al.* (1951) used a rat "stabilimeter", consisting of modified postage scales. A platform and confinement box were attached to the scales and, when rats were placed inside the box, any sharp sound induced a startle reaction which resulted in downward movement of the platform, deflection being measured via an ink polygraph. The conditioned stimulus (CS) consisted of a paired buzzer and light, the unconditioned stimulus (UCS) consisted of an electric shock (60 cycles, 70 - 80 V) applied via the grid forming the base of the confinement box, and the startle stimulus comprised the firing of a toy pistol. A four day training period was undertaken whereby, on each day, rats were trained through paired presentation of the CS and either the UCS (seven out of ten trials) or startle stimulus (three out of ten trials) - the CS was presented for 5 s and the UCS or startle

stimulus was presented for the final 2 s of the test period. Subjects demonstrated a significant increase in magnitude of startle response with each successive training day, indicating acquisition of a conditioned fear reaction. Subsequently, during three successive days of extinction trials, rats were presented with two paired presentations of the CS and UCS, followed by 12 presentations of the CS alone. The startle stimulus was presented 3 s after the onset of the CS in each trial (that is, at the same time at which the UCS would usually be presented). Subjects demonstrated a progressive decline in startle response magnitude with each successive non-reinforced trial (that is, presentation of the CS without the shock UCS) on each day, yet their startle responses had spontaneously recovered 24 h later. The authors concluded that rats trained to respond to a paired presentation of a neutral and a noxious stimulus demonstrated acquisition of emotional responses of "fear" or "anxiety" (conditioned fear reaction). Furthermore, presence of conditioned fear served to increase the intensity of response to the startle stimulus (Brown *et al.*, 1951), thus termed "fear-potentiated startle" (see Richardson, 2000).

(2) Punished responding

Punished responding (conflict) paradigms include the Geller-Seifter and Vogel tests. "Punishment is defined as the presentation of a noxious stimulus and results in the suppression of behaviour. This suppressive effect of punishment is seen clearly in conflict situations where the organism is encouraged to respond and then is punished for doing so" (Tye *et al.*, 1977). The Geller-Seifter test examines the conditioning of punishment discrimination and "conflict" behaviour, whereby rats are rewarded with food while simultaneously being punished by an electric shock (see Geller *et al.*, 1962). Geller *et al.* (1962) deprived rats of food, being reduced to 60 % of their original body weight, prior to testing. Subjects learned to press a lever in order to receive a liquid food reward delivered, on average, once during each 2 min period (variable interval; VI). Subsequently, an auditory stimulus (3 min tone) was presented at 15 min intervals during a 75 min test period. Each auditory cue denoted a change from the 2 min VI to a continuous reinforcement schedule (crf), where all presses of the lever resulted in delivery of food. After approximately seven such training periods, punishment was administered in the form of an electric shock of varying intensity, delivered via the grid

floor of the test box each time the lever was pressed during the presentation of the auditory stimulus (Geller *et al.*, 1962). Thus the animals experienced a "conflict" between seeking the food reward and receiving an electric shock, resulting in punishment discrimination behaviour whereby the animal "chooses" whether to receive the shock in order to obtain food. Lever-press responding is subsequently reduced with increasing intensity of electric shock.

This test can be used in the investigation of putative anxiolytic compounds. For example, Geller *et al.* (1962) administered chlordiazepoxide or chlorpromazine at several different doses and tested their effects on the lever-press responding of subjects administered high or low intensity shocks. Chlorpromazine treatment (1 mg/kg) resulted in a reduced number of shocks accepted at a low shock intensity and had no effect on responding at a high shock intensity, while chlordiazepoxide treatment (10 mg/kg) significantly attenuated punished discrimination at a high shock intensity (that is, the rats accepted more shocks in order to receive the reward) and had no significant effect at a low shock intensity. This study highlighted some of the differences between different classes of compounds, or those with differing mechanisms of action, as the anti-anxiety effect of chlordiazepoxide in the punishment discrimination paradigm was reported to be similar to the effects of meprobamate, phenobarbital and pentobarbital ("central nervous system depressants"), while the effect of chlorpromazine was reported to be similar to the effects of the phenothiazines, promazine and trifluoperazine (Geller *et al.*, 1962).

Kennett *et al.* (2000) employed a reinforcement schedule of ten 3 min tests which alternated between VI components, where one reinforcement was presented every 10 - 50 s, and fixed ratio (FR) components, comprising one reinforcement for every five lever presses. The FR component was accompanied by a light cue and reinforcement was paired with an electric foot shock (15 ms pulse at 200 ms intervals for 1 s), thus forming a punished responding trial. Punished responding was markedly increased by the benzodiazepine, chlordiazepoxide, whereas the novel 5-HT_{2C} receptor agonist, Ro 60 0175, significantly reduced both punished and unpunished responding. The authors

concluded that Ro 60 0175 was neither anxiolytic nor anxiogenic at the doses tested, but was markedly sedative (Kennett *et al.*, 2000).

The Vogel conflict test involves a similar principle to the Geller-Seifter test, where thirsty animals seeking a water reward are punished with an electric shock (see Vogel *et al.*, 1971). Vogel *et al.* (1971) deprived rats of water for 48 h prior to testing them in an apparatus consisting of two connected compartments with a stainless steel grid floor. The larger compartment comprised a clear Plexiglas box (38 x 38 cm) with an opening into a smaller compartment which comprised a black Plexiglas box (10 x 10.5 cm). A water bottle drinking tube extended into the smaller box and licks were recorded by the completion of a "drinkometer" circuit which connected the tube and the grid floor. Each subject was placed in the apparatus 30 min after drug administration and, upon finding the drinking tube, was allowed 20 licks before a shock was administered. The shock was terminated when the subject withdrew from the tube and a 3 min timer was activated at the end of the first shock. During the subsequent 3 min period, shocks were delivered after every 20 licks. This paradigm did not require training of the subjects, which removed any potential effects of repeated drug administration. Chlordiazepoxide pretreatment resulted in a dose-related increase in the number of shocks accepted during a 3 min test. Diazepam, oxazepam, meprobamate and pentobarbital all had similar effects, subjects showing a marked increase in punished responding in each case. These results were therefore in agreement with those of Geller *et al.* (1962), observed in the Geller-Seifter conflict paradigm (Vogel *et al.*, 1971).

Kennett *et al.* (1998; 2000) tested a group of rats which had been water-deprived for 20 h. Rats were subsequently placed in an operant conditioning chamber, with access to a water bottle spout, and allowed to freely explore and drink for 3 min, timed from the first lick of the bottle spout. Upon being returned to their home cage, rats were given 4 h free access to water, then deprived for a further 20 h. On the following day, subjects were placed in the test chamber and, after 30 s of continuous drinking from the bottle spout, subsequent 5 s drinking periods were punished by an electric shock (0.25 mA for 200 ms) delivered through the spout. The latency to begin licking again following each shock, and the total number of shocks administered, were recorded during a 3 min test.

Administration of the benzodiazepine, chlordiazepoxide (Kennett *et al.*, 1998; 2000), or the 5-HT_{2B} receptor agonist, BW 723C86 (Kennett *et al.*, 1998), significantly increased punished responding, whereas the 5-HT_{2C} receptor agonist, Ro 60 0175, had no effect (Kennett *et al.*, 2000).

3.1.1.2 Unconditioned tests

Unconditioned tests measure spontaneous, primarily exploratory, behaviour and include the light/dark test box, social interaction tests, elevated plus-maze and open-field (see Chaouloff *et al.*, 1997; Rodgers, 1997). The elevated plus-maze and open-field have been employed in the current investigation and therefore are described in greater detail in sections 3.1.2 and 3.1.3.

Unconditioned behaviour tests involve exploration of novel environments and are frequently used in the investigation of the behavioural effects of pharmacological agents. However, attention must be paid to locomotor activity, as this variable can impinge on the behavioural effects of drugs under investigation. Factor, or principal component, analysis is often used in an attempt to separate locomotion-dependent from locomotion-independent behaviours (Chaouloff *et al.*, 1997). Factor analysis describes the relationships between variables, aiding interpretation of several behavioural parameters within a single test or between a battery of tests (File, 1992). For example, on the elevated plus-maze, Factor 1 may refer to locomotor activity (closed and total arm entries, rearing) and Factor 2 may refer to approach/avoidance conflict (anxiety-related) behaviour (% open arm entries and % time spent on the open arms) (Chaouloff *et al.*, 1997; File, 1992). Additional factors may take into account exploratory behaviours, such as head-dipping (File, 1992).

(1) Light-dark test box

The light-dark (or black and white) test box is used to examine the conflict between exploratory behaviour and aversion to a brightly illuminated environment (see File, 1992). Costall *et al.* (1989) employed this paradigm to investigate the effects of a number of putative anxiolytic and anxiogenic compounds, administered 40 - 45 min prior to testing, in several strains of mice (albino BKW, black C57/BL/6, brown DBA_2

and albino Tuck). The apparatus comprised a two-compartment metal box (45 x 27 x 27 cm), the base of which was divided into 9 cm squares. The smaller compartment (two-fifths of the box) was painted black and always illuminated by a 60 W red light bulb, while the adjoining, larger compartment was painted white and illuminated by a white light bulb of varying intensity (15, 25, 40, 60, 100 W) or by a 60 W red light bulb. Following acclimatisation to the test room for 1 h, subjects were placed in the centre of either the white or the black compartment and observed for: (1) the number of exploratory rearings and line crossings within each compartment, (2) the number of transitions between the 2 compartments, (3) the time spent in the different compartments, and (4) the latency of the initial movement into the opposite compartment. Control data was obtained by placing mice in a test box illuminated solely by red light bulbs; these subjects demonstrated random activity between the two compartments which reflected the relative sizes of the sections, mice spending 60 - 70 % of the time in the larger section. There was no effect of changed illumination on the number of transitions performed by mice initially placed in either the white or the black arena. Increased illumination (60 and 100 W) significantly decreased the latency of movement from white to black and the % time spent in the white compartment, in mice initially placed in the white arena. Mice initially placed in the black compartment demonstrated a significantly greater latency to enter the white arena and spent significantly more time in the black section. Both rearing behaviour and the numbers of line crossings in the white section were significantly decreased in mice initially placed in either compartment, where there were high levels of illumination (60 and 100 W) in the white arena. Line crossings were significantly increased in the black compartment in mice initially placed in either section, while rearing behaviour in the black section was significantly increased in mice initially placed in this arena. Thus, the authors concluded that a brightly illuminated white arena was aversive and inhibited the exploratory behaviour of the mice (Costall *et al.*, 1989).

Aversion to a brightly illuminated environment, shown by reduced rearing behaviour, line crossings and time spent in the white arena, can be reversed by anxiolytic compounds (Chaouloff *et al.*, 1997; Costall *et al.*, 1989; Sánchez, 1995) so that the behaviour of drug-treated mice is the same as that of control mice (Costall *et al.*, 1989).

For example, the benzodiazepines diazepam, chlordiazepoxide and triazolam, the substituted benzamides sulpiride and tiapride, the 5-HT_3 receptor antagonists GR 38032F, ICS-205-930, MDL 72222, zacopride and BRL 43694, buspirone, alcohol, nicotine and the dopamine receptor antagonist SCH 23390 were shown to antagonise the aversion of the subjects to the brightly illuminated compartment. In contrast, FG7142, a benzodiazepine receptor inverse agonist, demonstrated anxiogenic properties shown by a reduced latency of movement into the black section and increased time spent, increased rearing behaviour and line crossings in the black compartment (Costall *et al.*, 1989).

(2) Social interaction

Social interaction tests examine the anxiety that is generated by placing rats in an unfamiliar and/or brightly illuminated environment (see File, 1992). Rats are housed individually for several days before the testing period. Under either low or high illumination, the subjects are then: (1) familiarised with the test box, by being placed individually in the box for 10 min on the two days immediately before the test day, or (2) given no prior acquaintance with the box. On the test day, randomly selected pairs of rats with the same previous familiarisation experience are placed in the test box for 10 min and monitored for interaction behaviours, such as sniffing, grooming, following, mounting and crawling over or under the other rat. Social interaction is maximal when rats are placed in a familiar environment under low levels of illumination. Increasing levels of illumination or unfamiliarity with the test box reduces levels of social interaction, this behaviour thus being minimal when rats are placed in a brightly lit, unfamiliar environment. Social interaction behaviour is sensitive to the effects of chlordiazepoxide; chronic pretreatment with the benzodiazepine has been shown to significantly reduce the unfamiliarity- and high illumination-induced decrease in this behaviour (File & Hyde, 1978; File, 1980).

(3) Activity meter

A simple measure of general locomotor activity, which is also measured as part of the plus-maze and open-field paradigms, is the activity meter. This model may comprise a clear plastic box with infrared beams, housed within a sound-attenuated cupboard.

Horizontal and vertical locomotor activity is measured by the number of beams crossed during a given period (see Griebel *et al.*, 1997b).

3.1.2 The elevated plus-maze

The elevated plus-maze is a widely used rodent model of anxiety-related behaviour, being employed in the development of anxiolytic drugs and as a method for attempting to understand the psychological and neurochemical bases of anxiety (see Dawson & Tricklebank, 1995). It is an approach/avoidance conflict model, as the natural exploratory behaviour of rodents in a novel environment ("approach", "curiosity") is in conflict with the animal's instinct to avoid aversive environments ("fear", "caution"), such as open spaces (see Figure 3.1; Handley & McBlane, 1993a; Handley *et al.*, 1993; Holmes, 2001).

The model was developed from an elevated Y-maze, which consisted of a total of three open and enclosed arms present in different ratios (0:3, 1:2, 2:1 or 3:0) (Montgomery, 1955). Female Wistar rats were tested in randomised order, each being placed individually in the centre of the Y-maze and allowed 10 min for free exploration, during which the sequence of maze arms entered was recorded. This process was repeated on successive days during a three or five day period (Montgomery, 1952; 1955; Montgomery & Monkman, 1955). It was demonstrated that: (1) exploratory behaviour decreased during each trial but recovered within 24 h (Montgomery, 1952), (2) that a novel environment could evoke both exploratory behaviour and "fear" (approach-avoidance conflict behaviour), and (3) that open arms evoked greater avoidance behaviour, indicative of greater "fear", than enclosed arms (Montgomery, 1955). In a further experiment, Montgomery & Monkman (1955) subjected the rats to an auditory stimulus to evoke a "fear" response. Evocation of a "fear" response immediately prior to testing on the Y-maze had no effect on the exploratory behaviour of the rats, while evocation of a "fear" response during the test reduced the amount of exploratory behaviour, demonstrating that exploratory behaviour is not motivated by "fear" (Montgomery & Monkman, 1955).

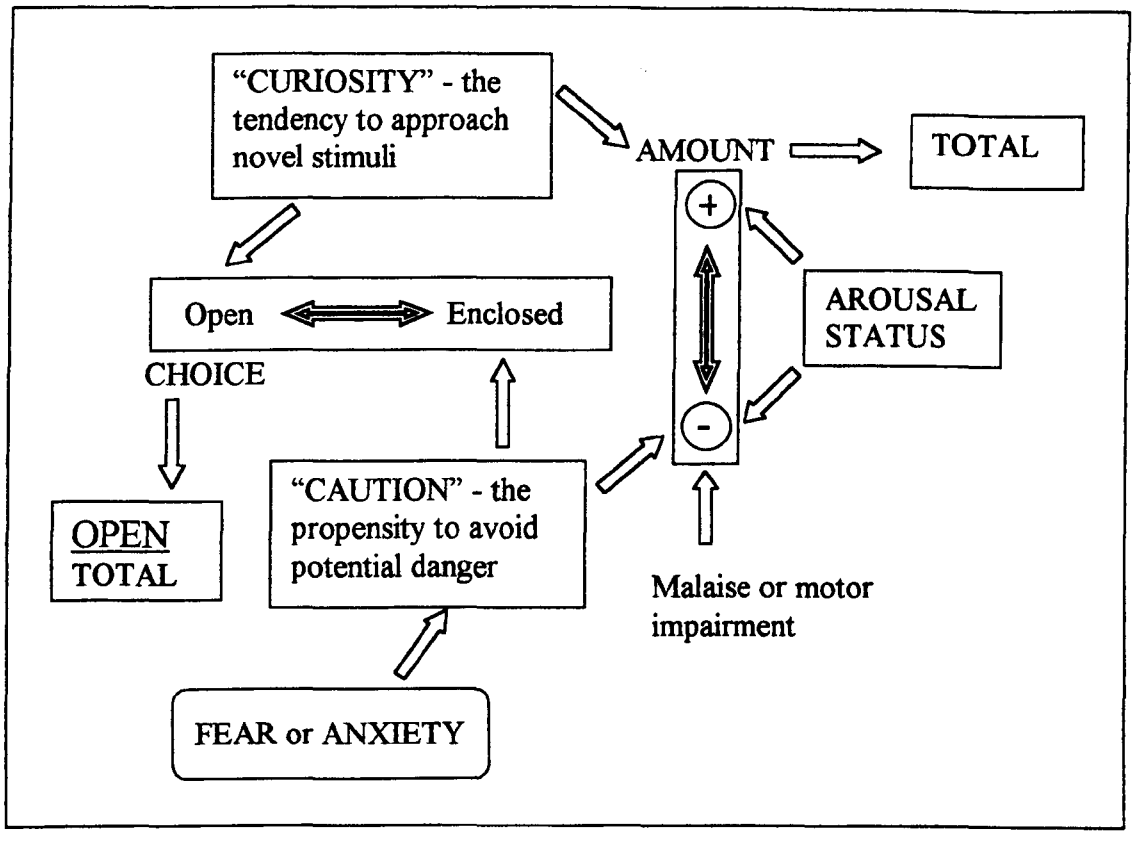

Figure 3.1 A schematic diagram of the theoretical basis of the elevated plus-maze (after Handley & McBlane, 1993a).

Novel stimuli induce both "curiosity" (approach), which results in open arm entries, and "caution" (avoidance), which results in enclosed arm entries. The level of fear or anxiety will impact on the degree of caution expressed. The open/total arm entries ratio expresses the outcome of the approach/avoidance conflict. Arousal status will differ with the administration of drugs, resulting in greater or lesser total movement, while motor impairment will reduce total activity.

Handley & Mithani (1984) constructed a plus-maze which was elevated 70 cm above the floor and consisted of two open and two enclosed arms (each 10 x 45 cm) positioned opposite to each other, the enclosed arms having 10 cm high walls. Male hooded Lister (HL) rats were tested for a 10 min period and the number of open and enclosed arm entries recorded. Rats behaved in a similar manner to that demonstrated in the Y-maze (Montgomery, 1955), demonstrating a clear preference for the enclosed arms of the maze. In particular the number of open arm entries, by being calculated as a ratio to total arm entries, was deemed to account for overall exploratory activity and provided a realistic measure of "fear"-motivated behaviour (Handley & Mithani, 1984). Open arm entries are usually expressed as a proportion of total arm entries, in an effort to remove any drug effects which affect both arm types equally (Handley & McBlane, 1993a). Pellow *et al.* (1985) further validated the elevated plus-maze as an animal model of anxiety, feeling the need for a short, simple test which relied on spontaneous activity, rather than requiring training procedures, noxious stimuli or food or water deprivation, such as the conditioned tests described above (see Brett & Pratt, 1990; Pellow *et al.*, 1985). Pellow and colleagues (1985) thus confirmed the results of Handley & Mithani (1984), in addition to identifying an increase in anxiety-related behaviours, such as freezing and defecation, on the open arms.

It has been assumed that avoidance of the open arms is due to fear of the height of the maze and/or high light intensity (see Dawson & Tricklebank, 1995), but fear of open space or lack of thigmotactic cues, where rats remain close to vertical surfaces, may be the primary anxiogenic factor (see Treit *et al.*, 1993). This has been demonstrated by varying the height of the maze (6, 25 and 50 cm above the floor), which was expected to alter open arm activity, but in fact there was no difference in the % number of open arm entries or % time spent on the open arms, between the different maze heights (Treit *et al.*, 1993). Furthermore, Handley & McBlane (1993a) tested Wistar rats on the elevated plus-maze in complete darkness (0 lux), observed via an infrared camera, and reported no difference in the open:total arm entries ratio compared to animals tested under normal lighting (170 lux).

Measurement of ethological variables can provide further indications of anxiety-like and exploratory behaviour and are important when assessing the effects of pharmacological manipulation on behaviour measured on the elevated plus-maze (see Setem *et al.*, 1999). For example, "scanning" or "head-dips", whereby the rodent extends its head over the edge of an open arm; "risk assessment", which involves extending the head and forepaws on to an open arm and may be accompanied by a "stretched attend posture" (i.e. full body stretch); "self-grooming", whereby the rodent cleans itself with the tongue, teeth and/or forepaws; "end-exploring", which is described as the number of times the rodent reaches the end of an open arm (Cruz *et al.*, 1994; Dawson & Tricklebank, 1995; Rodgers & Dalvi, 1997).

The elevated plus-maze test has been shown to be sensitive to the effects of anxiolytic and anxiogenic drugs. In particular, consistent and reproducible data has been reported on the behavioural profiles of compounds acting at the $GABA_A$-benzodiazepine receptor complex (see Hogg, 1996). Pretreatment with both the clinically effective anxiolytics chlordiazepoxide and diazepam (Pellow *et al.*, 1985) and the novel putative anxiolytics CL 218,872 and tracazolate (Pellow & File, 1986) resulted in increased exploration of the open arms, shown by an increased % number of open arm entries and % time spent on the open arms. Pretreatment with the known anxiogenic drug yohimbine (Pellow *et al.*, 1985) and the putative anxiogenic compounds FG 7142 and Ro 5-4864 (Pellow & File, 1986) led to reduced open arm exploration. Ethological variables further enable the detection of anxiolytic or anxiogenic properties of compounds, sometimes providing information which measures of locomotor activity, such as numbers of open arm entries, do not uncover (Setem *et al.*, 1999; Weiss *et al.*, 1998). For example, chlordiazepoxide significantly increased the % time spent on the open arms and numbers of head-dips, and decreased the numbers of stretched-attend postures (Weiss *et al.*, 1998), while the $5\text{-}HT_{1A}$ partial agonist ipsapirone was demonstrated to have anxiolytic properties by decreasing risk assessment and increasing scanning behaviour, without altering the % number of open arm entries or % time spent on the open arms (Setem *et al.*, 1999).

The plus-maze has also been demonstrated to be a suitable experimental paradigm for testing anxiety-related behaviours and the effects of anxiolytic and anxiogenic drugs in mice (see Lister, 1987). Lister (1987) tested male NIH Swiss mice on apparatus comprising two open (5 x 30 cm) and two enclosed (5 x 30 x 15 cm) arms, elevated 38.5 cm above the floor. Mice were placed in the centre of the maze, facing an open arm, and observed for 5 min. The total number of arm entries, % time spent on the open arms and % number of open arm entries were recorded. As seen in rats, mice were shown to have a consistent preference for the closed arms of the plus-maze. FG 7142 and caffeine treatment led to a reduced % time spent on the open arms, indicating an anxiogenic action. Chlordiazepoxide, ethanol and sodium pentobarbital increased both the % time spent on the open arms and the total number of arm entries, indicating anxiolytic actions (Lister, 1987).

Use of the elevated plus-maze as an effective model for investigating the neurotransmitter systems involved in anxiety-like behaviour is described in section 3.1.6.

Although the elevated plus-maze is extremely widely used, the results obtained are not always consistent, particularly concerning the use of serotonergic compounds (Graeff *et al.*, 1996; Handley & McBlane, 1993b). A possible explanation for such inconsistencies was described by Schwarting *et al.* (1998), whereby male Wistar rats were tested on the elevated plus-maze on two occasions, 24 h apart and then grouped according to "high-" or "low-anxiety" levels, as demonstrated by the amount of time spent on the open arms. Subsequent post-mortem analysis of 5-HT, dopamine and their metabolites showed reduced levels of 5-HT in the ventral striatum in the "high-anxiety" group, following the second plus-maze exposure. The ventral striatum includes the nucleus accumbens, an area associated with motivation; based on their data the authors suggested that this brain region may also be involved in emotional behaviour, and individual differences in serotonergic activity within the ventral striatum may help to explain the observed differences in animals' behavioural responses to pharmacological manipulation (Schwarting *et al.*, 1998).

The plus-maze has substantial construct validity as a model of anxiety, in that there is a high degree of similarity between its theoretical background and "anxiety" as a physiological state (see Handley & McBlane, 1993a) and the anxiolytic effects of a broad range of compounds, proven to be clinically effective, have been successfully demonstrated using this model. However, although the elevated plus-maze is commonly used as a screening method for compounds with anxiolytic potential (see Hogg, 1996; Treit, 1985), its predictive validity, or its ability to predict whether novel compounds will prove to be clinically effective, is still being proven (see Handley & McBlane, 1993a). In addition to its use as a model for studying the effects of specific compounds, the elevated plus-maze is useful for investigation of the neural mechanisms involved in the "anxiety" state itself (see File, 1992; Handley & McBlane, 1993a; Treit, 1985).

3.1.3 The open-field

A second, commonly used test of anxiety-related behaviour is the open-field, which has been used to examine "emotionality" or, " the state of being emotional...consists of a group of organic, experiential and expressive reactions and denotes a general upset or excited condition of the animal" (Hall, 1934b). Hall (1934a) used a circular open-field, which was 8 feet in diameter with a sheet tin wall 18 inches high. The base of the open field was linoleum and marked out in a number of zones and sections to enable tracking of the rat's movements. Rats were tested for 2 min, and defecation and urination were used as measures of "emotionality". "Emotional", rather than "normal", defecation and urination were defined as responses which ceased upon repeated exposure to the environment which originally evoked them, as rats placed in a novel environment demonstrated reductions in defecation and urination with successive trials (Hall, 1934a).

An additional measure of "emotionality" was the presentation of a food receptacle containing wet mash, placed in the centre of the open-field. The subjects had been food-deprived for 23 h and the number of trials (14 in total) required before each subject ate the food was seen as an indicator of the animal's level of "emotionality", the emotional response being a barrier to the satisfaction of the animal's need for food (Hall, 1934a; 1934b). Hall later investigated the relationship between "emotionality" and ambulatory activity. He tested rats in the open-field for 2 min every day for 28 successive days,

which varied with respect to food deprivation and presentation of food in the open-field. "Emotionality" was shown to correlate negatively with ambulatory activity; the more emotional the animals were (shown by increased defecation) the less active they were (Hall, 1936; Whimbey & Denenberg, 1967).

The open-field test also involves the conflict between the animal's exploratory drive and avoidance of open, brightly lit spaces (see Schmitt & Hiemke, 1998a); "anxiety" may override the animal's tendency to explore (see Zimmermann *et al.*, 2001). The apparatus tends to be highly illuminated (see Schmitt & Hiemke, 1998a; Zimmermann *et al.*, 2001) and may be either square (see Köhler & Lorens, 1978; Schmitt & Hiemke, 1998b), or circular (see Zimmermann *et al.*, 2001) and the base is divided into sections. Rats are placed in the open-field arena and observed for a set time period, ranging from 5 (see Escorihuela *et al.*, 1999) or 10 min (see Schmitt & Hiemke, 1998a; Zimmermann *et al.*, 2001) to 50 min (see Köhler & Lorens, 1978), and a number of behaviours are noted, including: numbers of squares entered, time spent in the inner and outer zones, rearing, grooming and defecation (see Escorihuela *et al.*, 1999; Köhler & Lorens, 1978; Schmitt & Hiemke, 1998b; Schwarting *et al.*, 1998; Zimmermann *et al.*, 2001). Furthermore, "enriched" environments may be employed as an additional measure of exploratory activity, whereby novel objects, such as plastic toys, are placed in the arena and approaches towards the objects and time spent sniffing the objects are recorded (see Schmitt & Hiemke, 1998b; Zimmermann *et al.*, 2001).

Similar to the elevated plus-maze, the open-field can be used for testing the anxiolytic or anxiogenic properties of different compounds (see Schmitt & Hiemke, 1998b), in addition to the effects of central serotonergic lesions. For example, electrolytic lesioning of the median raphe nuclei, approximately three weeks prior to behavioural testing, resulted in a 55 % reduction in forebrain 5-HT levels and increased locomotor activity within the open-field (Köhler & Lorens, 1978).

3.1.4 Effects of repeated testing and handling and duration of test

There is some evidence that repeated testing and/or handling alter rats' behavioural responses to pharmacological manipulation when tested on the elevated plus-maze. In

addition, different studies report different effects of repeated testing on control animals. For example, Bertoglio & Carobrez (2000) tested male Wistar rats on a series of modified plus-maze apparatus on two occasions, 48 h apart. They demonstrated a significant reduction in the % number of open arm entries and % time spent on the open arms of the standard elevated plus-maze in Trial 2, while enclosed arm entries (locomotor behaviour), % time spent in the centre of the maze and risk assessment behaviour were unaffected. The results in the elevated T-maze (two open arms, one enclosed arm) and L-maze (one open arm, one enclosed arm), whereby Trial 1 experience of these apparatus still resulted in reduced open arm behaviour in Trial 2, led the authors to suggest that the presence of at least two different aversive environments was the critical factor in the avoidance learning process. This was further confirmed by the lack of effect of open arm confinement in Trial 1 on the % number of open arm entries and % time spent on the open arms in Trial 2 (Bertoglio & Carobrez, 2000).

However, other authors have reported a lack of habituation with repeated testing. For example, Pellow *et al.* (1985) tested a group of male HL rats on the plus-maze on three consecutive days, examining the numbers of open and enclosed arm entries and the time spent on open and enclosed arms. Habituation did not occur, as there was no significant difference in the % number of open arm entries, the % time spent on the open arms, or the total number of arm entries, across the three test days, demonstrating that novelty was not a critical factor in the rats' behaviour (Pellow *et al.*, 1985). Lister (1987) demonstrated no change in anxiety-related behaviours (% open arm entries and % time spent on the open arms) in mice tested on the elevated plus-maze on four occasions, 48 h apart. Treit *et al.* (1993) reported findings consistent with those of Pellow *et al.* (1985), where male SD rats were tested on the elevated plus-maze on 18 consecutive days and demonstrated no significant change in the % time spent on the open arms, indicating a lack of habituation. However, there was a marginal decrease in the % number of open arm entries between the first and last trials, which may have indicated an increase in "fear" of the open arms after the first trial (Treit *et al.*, 1993). These results were further explored by confining the subjects to an open arm of the plus-maze for 30 min on three consecutive days, followed by free exploration of the apparatus for 5 min on each of the following three days. It was expected that forced exposure to the

open arms would lead to reduced avoidance of these areas, but instead resulted in decreased % time spent on the open arms and % number of open arm entries, compared to control animals, during the first of the subsequent free exploration trials. Thus, it appeared that rats' "fear" of open spaces was resistant to modification (Treit *et al.*, 1993). Handley & McBlane (1993a) investigated whether lack of habituation was due to rats having insufficient time, during a 10 min plus-maze exposure, to fully evaluate their environment. Thus, they tested Wistar rats on the elevated plus-maze for 1 h, then on the following day examined behaviour during a 10 min exposure. Total arm entries were marginally decreased on Day 2 compared with the first 10 min on Day 1, while open arm entries decreased by 74 % and open/total arm entries ratio decreased by 58 % on Day 2, indicating that prolonged exposure to the plus-maze led to an increase in open arm aversion (Handley & McBlane, 1993a).

File *et al.* (1990) also demonstrated that control animals do not differ in their responses on the elevated plus-maze over repeated tests, while a single previous trial ranging from 24 h (File, 1990) to two weeks earlier (File *et al.*, 1990), renders the animal insensitive to the anxiolytic effects of chlordiazepoxide and is termed "one-trial tolerance". This phenomenon is believed to involve an interaction between the animal's experience on the elevated plus-maze during the first trial and an action of chlordiazepoxide with benzodiazepine receptors during the second trial. Chronic treatment is usually required to induce tolerance to chlordiazepoxide-induced anxiolysis on the elevated plus-maze, but a single 5 min prior exposure to the maze has the same behavioural effect (File, 1990; File *et al.*, 1992). The animal's experience of the open arms of the elevated plus-maze during Trial 1 was thought to be the crucial factor in this phenomenon (File *et al.*, 1990).

In a later study, File *et al.* (1992) demonstrated an additional effect of handling on the responses of chlordiazepoxide-treated rats. Male HL rats were tested on the elevated plus-maze; one group were handled and injected with saline daily for three weeks and a second group were not handled until the experimental day. Within each group, animals were administered either chlordiazepoxide or vehicle 30 min prior to being tested on the plus-maze and all groups were further divided into those which were tested on a single

occasion and those which were tested on two successive days. In unhandled animals, chlordiazepoxide significantly increased the % time spent on the open arms both in animals with no prior maze experience and in animals which had been tested on the elevated plus-maze on the previous day. In handled animals, however, chlordiazepoxide still induced a significant increase in the % time spent in the open arms in maze-naïve animals, but this effect was abolished in the group which had previously been exposed to the plus-maze. The authors concluded that the effects of handling and prior experience of the plus-maze had a combined effect of reducing the anxiolytic effect of chlordiazepoxide (File *et al.*, 1992).

Andrews & File (1993) demonstrated effects of chronic handling in vehicle-treated male HL rats. Handling-habituated rats were subjected to daily handling for seven days, which consisted of weighing each rat and administering an i.p. injection of water. The unhandled rats were undisturbed until the test day. Vehicle-treated unhandled rats spent significantly less % time on the open arms and performed a significantly lower % number of open arm entries, compared to handled rats. However, the authors do state that these differences were not consistently reproducible and may be due to differences in handling history by the animal supplying company (Andrews & File, 1993). Brett & Pratt (1990) demonstrated no differences between handled and unhandled control animals, in their sub-acute study on male Long-Evans hooded rats, where the handling procedure involved being handled daily for two days, then being injected with diazepam or vehicle daily for three days. They demonstrated a significant increase in the ratio of open/total entries and time in the diazepam-treated rats, compared to the vehicle-treated rats. However, in a chronic experiment, where rats were handled daily for two days, then: (1) injected daily with vehicle for 27 days, or (2) injected with vehicle for 24 days then diazepam for three days, or (3) injected with diazepam for 27 days, there were no significant differences between treatment groups with respect to the ratio of open/total entries or time. The authors concluded that the lack of anxiolytic effect of diazepam in the chronic study was due to the chronic handling and injection regimen (Brett & Pratt, 1990).

The length of the plus-maze trials has also been demonstrated to affect the behavioural response of rats to diazepam. File *et al.* (1993a) tested male HL rats on the elevated plus-maze on two occasions, 24 h apart. Rats were administered diazepam or vehicle and tested for either 5 or 10 min on each occasion. Diazepam had an anxiolytic effect in the first trial, whether 5 or 10 min, shown by increasing the % number of open arm entries. The anxiolytic effect of diazepam was abolished in rats which were tested for 5 min in both Trial 1 and Trial 2; there was no difference in the % number of open arm entries between control and diazepam-treated animals. However, rats which were tested for 10 min in both trials still demonstrated an anxiolytic response to diazepam (File *et al.*, 1993a).

These data have led to the suggestion that different forms of "fear" may be expressed under different conditions. It may be that Trial 1 "anxiety" involves the unfamiliarity of the environment and "fear" of the open arms, while Trial 2 primarily involves "fear" of heights (File *et al.*, 1993a). However, the latter is now believed to be more likely the lack of thigmotactic cues, rather than the degree of elevation (Treit *et al.*, 1993).

Untreated mice have been reported to differ in their behavioural responses on the elevated plus-maze in successive trials. Male Swiss-Webster mice were tested on the plus-maze for 5 min, on three successive days (Trials 1 - 3) and Trial 4 was conducted ten days after the third trial. Mice demonstrated significant reductions in total arm entries, % number of open arm entries, % time spent on the open arms, head-dipping and stretched attend postures, in Trial 2 compared to Trial 1. These changes were maintained or further enhanced in Trial 3 and in Trial 4 (Holmes & Rodgers, 1998). Although mice which were not subject to any pharmacological manipulation differed in their responses in successive trials on the elevated plus-maze, the same responses to chlordiazepoxide were observed as those seen in rats. Chlordiazepoxide induced an anxiolytic response in maze-naïve mice, shown by an increase in % time spent on the open arms. This response was abolished in a second 5 min exposure to the plus-maze 24 h later, regardless of whether the first trial was of 5 min or 10 min duration. The latter effect was reversed in a 10 min trial, demonstrating that prior maze experience does not

affect responses to chlordiazepoxide when both trials are of 10 min duration (Holmes & Rodgers, 1999).

3.1.5 Behavioural testing in different strains

There is a lack of consistency in the results obtained in animal behavioural testing, due to differences in specific experimental conditions and laboratories (Handley & McBlane, 1993b; Hogg, 1996). Genetic (Ramos & Mormède, 1998) and gender (Palanza, 2001; Ramos & Mormède, 1998) differences can also induce different behavioural responses to environmental challenges.

The physiological mechanisms involved in "emotion" may be further clarified by characterising different strains with respect to a variety of behavioural measures (Ramos & Mormède, 1998). For example, male and female Lewis (LEW) and spontaneously hypertensive rats (SHR) were tested in the open-field, elevated plus-maze, activity meter and black and white box. With regard to locomotion, shown by total activity in the open-field, closed arm entries on the plus-maze and beam breaks in the activity meter, there were no differences between the two strains or between genders. However, with regard to anxiety-related behaviour, shown by reduced locomotor activity in the central zone of the open-field, % number of open arm entries and % time spent on the open arms of the plus-maze and time spent in the white compartment of the black and white box, the LEW rats were demonstrated to be more "anxious". In addition, male rats within both strains tended to demonstrate greater anxiety-related behaviours (Ramos & Mormède, 1998; Ramos *et al.*, 1998). The authors concluded that these two strains could prove a useful genetic tool in the study of anxiety (Ramos *et al.*, 1998).

Rex *et al.* (1999) compared the behaviour of male Fischer 344 and Harlan-Wistar rats on the elevated plus-maze, black and white test box, social interaction test and a modified (food reward) open-field test. The Fischer 344 rats showed increased anxiety-related behaviour compared to the Harlan-Wistar rats, which was consistent across all tests. The Fischer rats did not seek food in the modified open-field test, had a greater latency of contact and spent less time in social contact in the social interaction test, spent less time on the open arms and entered the open arms less frequently on the

elevated plus-maze, and spent less time in the white compartment of the black and white test box (Rex *et al.*, 1999). King (1999) compared the behaviour of three strains of rat - SD, Dark Agouti (DA) and HL - on the standard elevated plus-maze and on an unstable elevated plus-maze, which comprised four open arms and was oscillated in the horizontal plane. The DA rats displayed greater anxiety-related behaviour than the other two strains, spending less time on the open arms, performing fewer open arm entries and having lower overall locomotor activity (total distance travelled). The HL rats were shown to be least "anxious" with regard to these parameters. The HL rats were again less "anxious" on the unstable plus-maze, as they performed significantly fewer escape attempts than the other two strains (King, 1999). Pellow *et al.* (1985) compared the behaviour of two strains of rat, HL and Wistar, on the elevated plus-maze and demonstrated no significant differences. Both strains performed significantly fewer open arm entries and spent less time on the open arms compared to the enclosed arms, indicating that either strain would be suitable for testing on the elevated plus-maze. However, during their pilot studies the authors observed that black hooded PVG rats did not show a consistent aversion to the open arms. Therefore, they advocated care when choosing a suitable strain for this model (Pellow *et al.*, 1985).

In addition, genetic manipulations in mice provide models for the neural mechanisms of "anxiety" (Holmes, 2001). For example, Heisler *et al.* (1998) bred 5-HT_{1A} receptor null-mutant mice through truncation of the 5-HT_{1A} receptor protein. Therefore, the homozygous mutant mice lacked functional 5-HT_{1A} receptors, as indicated by receptor autoradiography and a lack of hypothermic response to 8-OH-DPAT administration. Mutant mice demonstrated increased "anxiety" levels, shown by significantly reduced locomotor activity and crossings within the central zone of the open-field, and significantly increased latency to respond to a novel object within the home cage (familiar environment), compared to both heterozygous and wild-type mice (Heisler *et al.*, 1998).

3.1.6 5-HT and anxiety

The role of 5-HT in anxiety-related behaviour has been investigated by disrupting central serotonergic activity or by administering 5-HT receptor-selective ligands, and

analysing subsequent behavioural responses in both cases. In addition, *in vitro* studies have aimed to elucidate whether pre- or post-synaptic mechanisms are involved. However, it is important to note that responses to 5-HT ligands in animal behavioural paradigms differ depending on the model, specific experimental conditions and the laboratory (Dawson & Tricklebank, 1995; Handley & McBlane, 1993b; Handley *et al.*, 1993; Schwarting *et al.*, 1998). Such differences in responses to pharmacological manipulation may indicate multiple mechanisms of anxiety (Graeff *et al.*, 1996; Handley *et al.*, 1993).

3.1.6.1 Disruption of central serotonergic activity

The selective serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) has been used in several behavioural paradigms to determine the effects of central 5-HT lesions on animal behaviour. For example, Briley *et al.* (1990) administered 5,7-DHT (250 μg) via an intracerebroventricular (i.c.v.) injection and measured 5-HT levels and [^{3}H]paroxetine binding 14 days later. A significant decrease in cortical (81 %) and hippocampal (99 %) 5-HT levels, in addition to reduced cortical [^{3}H] paroxetine binding (53 %), was observed. Anxiety-like behaviour was significantly reduced in the lesioned animals, as demonstrated by the increase in % number of open arm entries on the elevated plus-maze, while locomotor activity was unchanged (Briley *et al.*, 1990).

Hall *et al.* (1999) administered 5,7-DHT i.c.v. to male SD rats and subsequently measured 5-HT levels via an *in vivo* microdialysis probe implanted in the left striatum. Two weeks post-lesioning rats were tested on the elevated plus-maze and open-field. Rats were grouped according to the extent of 5-HT depletion (low: 0 - 50 %; moderate: 51 - 75 %; high: 76 - 100 %) and their behavioural responses analysed. Increased levels of anxiety were observed in animals with low or moderate 5-HT depletion, as shown by reduced numbers of crossings in the central zone of the open-field and reduced numbers of open arm entries and increased defecation in the plus-maze. However, high levels of 5-HT depletion significantly reduced crossings within the outer zone of the open field, had no effect on crossings within the central zone and no effect on behaviour in the elevated plus-maze. The authors suggested that serotonergic lesions may have a biphasic effect on anxiety, whereby increasingly severe lesions reversed the anxiogenic

effects of smaller lesions. Another possible explanation for this result was that compensatory changes were occurring after partial lesions and were abolished following complete lesions (Hall *et al.*, 1999).

In addition to altering responses in the elevated plus-maze and open-field, 5,7-DHT administration has been shown to increase punished responding (Tye *et al.*, 1977). Rats were trained to press a reinforcement lever to receive a food reward and, in subsequent tests, the reinforcement was paired with an electric foot-shock. Lesioned animals continued to press the reinforcement lever when shocks were administered, whereas control animals learned to avoid the foot-shocks. Thus 5-HT depletion appeared to have a "punishment-releasing effect", reversing the suppressive effects of punishment (Tye *et al.*, 1977).

Another compound employed to disrupt serotonergic activity is *p*-chlorophenylalanine (PCPA), which is an irreversible tryptophan hydroxylase inhibitor, and administration thus results in depletion of 5-HT (see Koe & Weissman, 1968). File & Hyde (1977) administered a single dose of PCPA (400 mg/kg i.p.) to male hooded rats three days prior to a social interaction test (see section 3.1.1). Brain 5-HT concentrations were depleted by 74 % while social interaction, which declines with increased illumination and levels of unfamiliarity (File & Hyde, 1978), was consistent over all test situations (low/high illumination and/or familiar/unfamiliar environment) (File & Hyde, 1977). PCPA has also been shown to increase punished responding in animals where a 51 % reduction in whole brain 5-HT concentration was recorded (Tye *et al.*, 1979). Such data indicated a role for 5-HT in the mediation of conflict behaviour (File & Hyde, 1977; Tye *et al.*, 1977; 1979).

3.1.6.2 5-HT receptor-selective ligands

5-HT-related compounds have been extensively studied on the elevated plus-maze. 5-HT_2 and 5-HT_3 receptor antagonists and 5-HT_{1A} partial agonists have anxiolytic actions, while 5-HT_2 and 5-HT_{1C} agonists have been demonstrated to be anxiogenic. However, different studies have reported different drug actions, effects also varying between anxiety models (see Handley & McBlane, 1993b). Griebel *et al.* (1997a) tested the

effects of several 5-HT receptor-specific antagonists on the behaviour of male SD rats in the elevated plus-maze. The non-selective 5-HT_2 antagonist mianserin and the selective 5-$HT_{2B/2C}$ antagonist SB 206553 were both demonstrated to have anxiolytic actions, as shown by increasing both the % number of open arm entries and the % time spent on the open arms, compared to control animals. The magnitude of response in both cases was similar to that following pretreatment with the benzodiazepine diazepam. The selective 5-HT_{2A} receptor antagonist MDL 100,907 had no effect on these parameters, indicating that blockade of this receptor does not have an anxiolytic-like effect in rats tested on the elevated plus-maze model of anxiety (Griebel *et al.*, 1997a). The 5-HT_{1A} receptor partial agonist buspirone was demonstrated to increase both the % time spent in the open arms and risk assessment (head-dipping) behaviour (Griebel *et al.*, 1997b). Similarly, buspirone and ipsapirone have been demonstrated to increase exploratory behaviour of mice in the white compartment of a light-dark test box (Sánchez, 1995).

3.1.6.3 *In vitro* studies

File *et al.* (1993b) investigated the effects of a social interaction test and elevated plus-maze test on the subsequent release and uptake of GABA and 5-HT in hippocampal and frontal cortical slices. Male HL rats were subjected to one of a series of test conditions: (1) remaining in the home cage, (2) a single 5 min plus-maze test, (3) two 10 min plus-maze tests separated by 24 h, or (4) a social interaction test with varying light and familiarity conditions. Brain tissue was obtained immediately after the test and 200 μm hippocampal and cortical slices prepared. Potassium-evoked release of [^{3}H]5-HT was significantly increased in hippocampal slices prepared from animals exposed to the 5 min elevated plus-maze test or the social interaction test (high illumination, familiar condition). These results provided some indication of rapid changes in presynaptic function, as a response to anxiety-testing (File *et al.*, 1993b).

3.1.7 Aims of the investigation

The longer-term behavioural consequences of MDMA administration in rats have been little studied and were therefore examined as part of this thesis (see Chapter 4). In addition, there is very little published data on the behaviour of the DA strain (apart from

King, 1999), which has been used in several earlier studies on the neurotoxic consequences of MDMA administration (see Colado *et al.*, 1995; O'Shea *et al.*, 1998) and was to be used for most studies reported in this thesis. Therefore, as a prelude to investigating the longer-term behavioural effects of MDMA administration in the DA strain, it was important to establish a baseline. This was achieved by comparing the locomotor and anxiety-related behaviour of DA rats with that of SD rats, a more commonly used strain (e.g. Briley *et al.*, 1990; Griebel *et al.*, 1997a; 1997b; King, 1999; Schmitt & Hiemke, 1998a; 1998b). Two widely used behavioural paradigms, the elevated plus-maze and open-field, were thus employed, in addition to the activity meter which provided further information concerning locomotor behaviour in the two strains.

Following the determination of baseline behaviours in both strains, the effects of acute diazepam administration were investigated. Diazepam is a clinically-effective anxiolytic compound which has been used in the characterisation of the elevated plus-maze as a suitable model of "anxiety" (see Handley & Mithani, 1984; Pellow *et al.*, 1985) and is frequently employed when testing animals on the plus-maze (e.g. Brett & Pratt, 1990; File *et al.*, 1993a; Treit *et al.*, 1993). Diazepam treatment induces a clear anxiolytic response, shown by increased open arm exploration (see Pellow *et al.*, 1985). Anxiolysis has also been demonstrated, in response to diazepam administration, in other behavioural models, such as the Vogel conflict test (Vogel *et al.*, 1971) and the light-dark test box (Costall *et al.*, 1989). This drug was employed in the current study as an additional measure for assessing the suitability of DA rats for future behavioural investigation.

3.2 METHODS

3.2.1 Animals and drug administration

Adult male DA and SD rats were housed as detailed in section 2.1(2). Diazepam was dissolved in 0.9 % NaCl w/v with 15 % Tween 20 and injected at a dose of 1 or 1.5 mg/kg s.c., 30 min prior to rats being tested on the plus-maze.

3.2.2 Behavioural assessment

DA and SD rats (n = 9, in each group) were tested on the elevated plus-maze in randomised order on two occasions: Day 1 and Day 26. The test days were deemed to be separated by a suitable time period to prevent the development of habituation. Animals were monitored for numbers of arm entries, time spent on open and closed arms and a number of simple behaviours, as detailed in section 2.6.1. All animals were also tested in the open-field, on the three consecutive days subsequent to the first plus-maze test day, being monitored for numbers of zone crossings and several anxiety-related behaviours (see section 2.6.2). Days 1 and 2 were performed under white-light illumination, to test any possible effects of habituation, and Day 3 was performed under red-light illumination.

A second group of DA and SD rats (n = 8, in each group) were tested in automated activity meters and monitored for overall locomotor activity (total numbers of infrared beam-breaks) and rearing activity (numbers of upper level beam-breaks). A further group of DA and SD rats were administered diazepam (1 mg/kg) or vehicle (n = 8, in each group) 30 min prior to being tested on the elevated plus-maze. Locomotor and ethological variables were monitored and recorded as before. This experiment was repeated two weeks later, when diazepam was again administered, but at the higher dose of 1.5 mg/kg.

3.2.3 Statistics

The elevated plus-maze and open-field data were analysed by two-way analysis of variance (ANOVA), with repeated measures, with STRAIN (DA or SD) x DAY (day of testing) as factors, followed by a Bonferroni post-hoc test. Activity meter data was analysed by two-way ANOVA, with repeated measures, with STRAIN (DA or SD) x TIME (of data collection) as factors, followed by a Bonferroni post-hoc test. The effects of diazepam were analysed by two-way ANOVA, with STRAIN (DA or SD) x TREATMENT (diazepam or vehicle) as factors, followed by a Bonferroni post-hoc test.

3.3 RESULTS

3.3.1 Behaviour of DA and SD rats on the elevated plus-maze

Naïve DA and SD rats demonstrated pronounced differences in their behaviour on the elevated plus-maze. With regard to anxiety-related behaviour, DA rats performed a significantly lower % number of open arm entries (Figure 3.2a) and spent significantly less % time on the open arms (Figure 3.2b) on both test days compared to SD rats, resulting in main effects of STRAIN. The % number of open arm entries and % time spent on the open arms did not differ between the two test days in either strain, thus no main effects of DAY or interactions were observed.

With regard to locomotor behaviour, DA rats were significantly less active than the SD strain on both test days, shown by lower numbers of total and closed arm entries. DA rats performed an approximately three-fold lower number of total arm entries on Day 1 and approximately two-fold lower number of total arm entries on Day 26 compared to SD rats, resulting in a main effect of STRAIN (Figure 3.2c). Similarly, DA rats performed approximately half the number of closed arm entries than SD rats on both test days, resulting in a main effect of STRAIN (Figure 3.2d). There were no differences in the numbers of total or closed arm entries between the two test days in either strain, therefore neither main effects of DAY nor interactions were observed.

With regard to the ethological variables measured on the elevated plus-maze (Table 3.1), DA rats performed significantly greater defecation and grooming behaviour compared to the SD strain. This resulted in main effects of STRAIN and, with respect to grooming behaviour, post-hoc analysis showed a statistically significant difference between strains on both Day 1 ($p < 0.05$) and Day 26 ($p < 0.001$). However, there were no differences within either strain when comparing both the defecation and grooming behaviour on the two test days, thus neither main effects of DAY nor interactions were observed in either case. DA rats performed significantly fewer numbers of head dips compared to SD rats, resulting in a main effect of STRAIN and post-hoc analysis showed a statistically significant difference on Day 1 ($p < 0.01$).

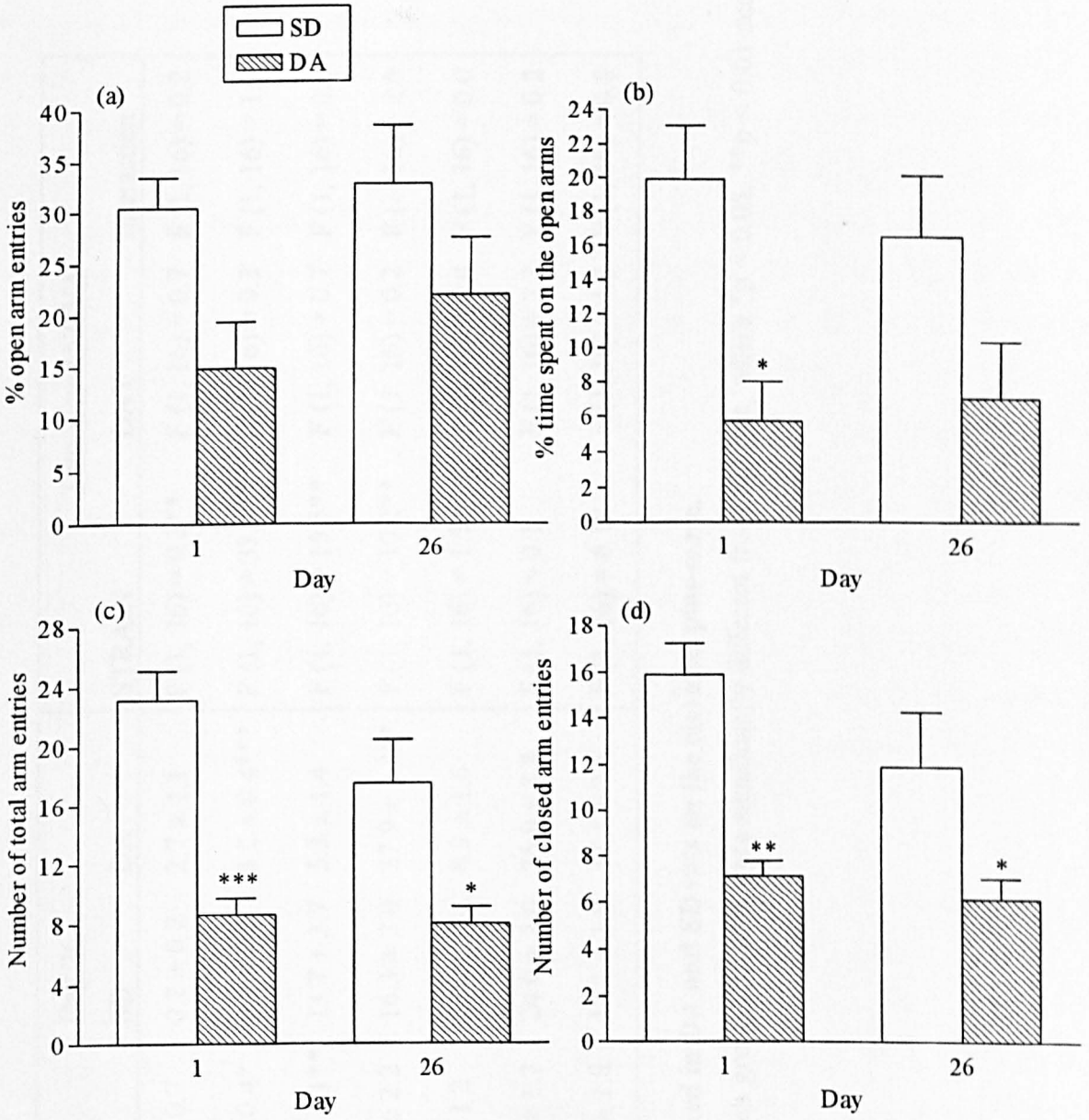

Figure 3.2 The behaviour of DA and SD rats on the elevated plus-maze.

Results shown as mean ± s.e.m., n = 9 in each group. ***(a)*** *Open arm entries, calculated as a % of total arm entries.* DA rats were different from SD rats ($F(1, 16) = 6.4$, $p < 0.05$). There was no difference between days within either strain. ***(b)*** *Time spent on the open arms, calculated as a % of time spent on all arms.* DA rats were different from SD rats ($F(1, 16) = 10.8$, < 0.01) with post-hoc analysis showing a significant difference between strains on Day 1 ($*p < 0.05$). There was no difference between days within either strain. ***(c)*** *Total arm entries.* DA rats were different from SD rats ($F(1, 16) = 29.0$, $p < 0.0001$) and post-hoc analysis demonstrated a significant difference between strains on both Day 1 ($***p < 0.001$) and Day 26 ($*p < 0.05$). There was no difference between days within either strain. ***(d)*** *Closed arm entries.* DA rats were different from SD rats ($F(1, 16) = 21.8$, $p < 0.001$) and post-hoc analysis demonstrated a significant difference between strains on both Day 1 ($**p < 0.01$) and Day 26 ($*p < 0.05$). There was no difference between days within either strain.

	Day 1		Day 26		Two-way ANOVA		
	SD	DA	SD	DA	STRAIN	DAY	Interaction
Defecation	0.0 ± 0.0	1.9 ± 0.7	0.2 ± 0.2	2.7 ± 1.1	F (1, 16) = 9.3**	F (1, 16) = 0.7	F (1, 16) = 0.2
Grooming	1.1 ± 0.3	2.4 ± 0.4*	0.8 ± 0.5	3.2 ± 0.4***	F (1, 16) = 33.3***	F (1, 16) = 0.3	F (1, 16) = 1.7
Head dips	13.4 ± 2.5	1.7 ± 1.1**	13.7 ± 3.7	5.3 ± 1.4	F (1, 16) = 15.5**	F (1, 16) = 0.7	F (1, 16) = 0.6
Rearing	21.7 ± 1.5	24.9 ± 2.2	16.3 ± 3.0	27.9 ± 2.8**	F (1, 16) = 10.2**	F (1, 16) = 0.2	F (1, 16) = 2.6
SAP	6.4 ± 1.0	8.2 ± 1.2	7.0 ± 1.3	8.9 ± 1.6	F (1, 16) = 1.5	F (1, 16) = 0.4	F (1, 16) = 0.0
Sniffing (directed)	21.3 ± 2.3	21.0 ± 1.7	24.0 ± 2.0	25.9 ± 3.8	F (1, 16) = 0.1	F (1, 16) = 2.1	F (1, 16) = 0.2
Sniffing (non-directed)	13.4 ± 1.2	20.2 ± 1.8	13.1 ± 1.8	17.1 ± 2.5	F (1, 16) = 6.1*	F (1, 16) = 1.2	F (1, 16) = 0.8

Table 3.1 Ethological variables measured in DA and SD rats on the elevated plus-maze.

Results shown as mean ± s.e.m., n = 9 in each group. DA rats were significantly different from SD rats, where *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

However, there was no difference between the number of head dips performed on Day 1 and Day 26 within either the DA or the SD group, therefore there was no main effect of DAY and no interaction. The DA rats performed significantly greater rearing behaviour which resulted in a main effect of STRAIN, and post-hoc analysis revealed a statistically significant difference between the two strains on Day 26 ($p < 0.01$). There was neither a main effect of DAY nor an interaction. SAP and directed sniffing behaviour did not differ between the DA and SD groups on either test day, while non-directed sniffing was significantly greater in DA rats, resulting in a main effect of STRAIN, but no main effect of DAY or interaction.

3.3.2 Behaviour of DA and SD rats in the open-field

The behaviour of naïve DA and SD rats was markedly different in the open-field. The number of Zone 2 crossings was significantly lower in the DA group compared to the SD rats (Figure 3.3a), resulting in a main effect of STRAIN and, although the number of Zone 2 crossings performed by the SD rats increased on consecutive days, there was no main effect of DAY and no interaction.

DA rats demonstrated significantly lower overall locomotor activity, compared to the SD group, as shown by an approximately three-fold lower number of total zone crossings on all three test days (Figure 3.3b), resulting in a main effect of STRAIN. However, when comparing responses on each of the three test days, there was no difference between the total number of zone crossings performed by either DA or SD rats, thus there was no main effect of DAY and no interaction.

With regard to the ethological variables measured in the open-field (Table 3.2), DA rats performed significantly greater defecation and grooming behaviour compared to SD rats. This resulted in main effects of STRAIN in both cases, and post-hoc analysis showed statistically significant differences on Days 2 and 3 ($p < 0.001$), with respect to defecation behaviour, and on Day 1 ($p < 0.05$) with respect to grooming. There were no differences within either strain when comparing defecation behaviour on the three test days, therefore there was neither a main effect of DAY nor an interaction.

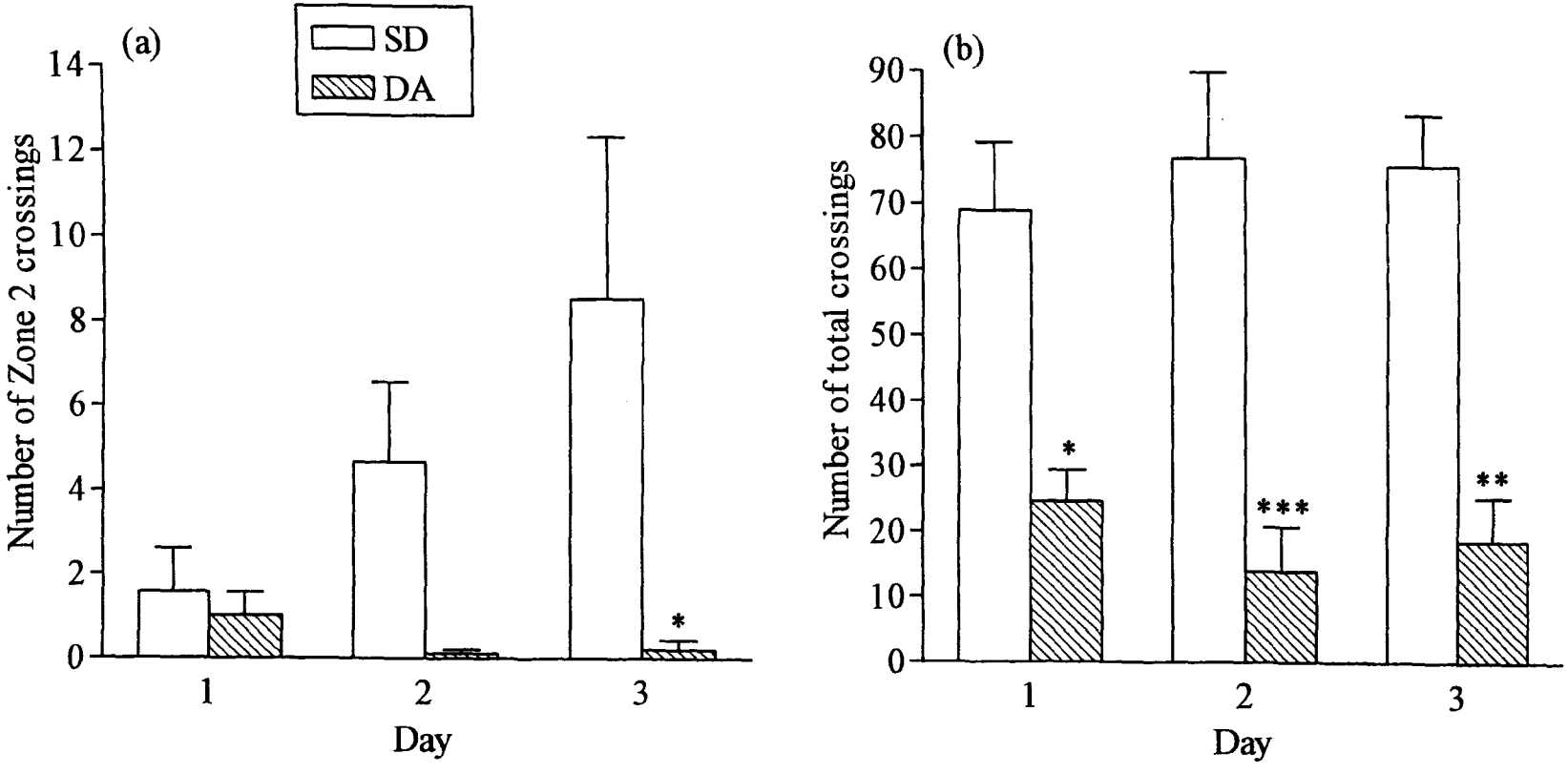

Figure 3.3 The behaviour of DA and SD rats in the open-field.

Days 1 and 2 were performed under white-light illumination and Day 3 was performed under red-light illumination. Results shown as mean ± s.e.m., n = 9 in each group. ***(a)*** *Zone 2 crossings.* DA rats were different from SD rats ($F(1, 24) = 8.1$, $p < 0.01$), while post-hoc analysis showed a statistically significant difference on Day 3 (*$p < 0.05$). There was no difference between days within either strain. ***(b)*** *Total number of zone crossings.* DA rats were different from SD rats ($F(1, 24) = 43.4$, $p < 0.0001$), while post-hoc analysis showed statistically significant differences on Day 1 (*$p < 0.05$), Day 2 (***$p < 0.001$) and Day 3 (**$p < 0.01$). There was no difference between days within either strain.

	Day 1		Day 2		Day 3		Two-way ANOVA		
	SD	DA	SD	DA	SD	DA	STRAIN	DAY	Interaction
Defecation	0.1 ± 0.1	1.6 ± 0.6	0.1 ± 0.1	3.7 ± 1.0***	0.2 ± 0.2	3.3 ± 0.7***	F (1, 24) = 40.7***	F (2, 24) = 1.8	F (2, 24) = 1.7
Freezing	4.2 ± 0.7	3.7 ± 0.5	2.9 ± 0.9	3.1 ± 0.6	4.6 ± 0.7	4.0 ± 0.6	F (1, 24) = 0.3	F (2, 24) = 1.7	F (2, 24) = 0.2
Grooming	0.4 ± 0.2	3.4 ± 0.7*	0.7 ± 0.3	2.4 ± 0.7	1.1 ± 0.4	3.6 ± 0.6	F (1, 24) = 18.5***	F (2, 24) = 3.5$^{\Delta}$	F (2, 24) = 2.2
Rearing	7.0 ± 1.7	11.0 ± 1.8	10.9 ± 2.2	6.0 ± 1.8	13.4 ± 1.5	16.3 ± 3.6	F (1, 24) = 0.1	F (2, 24) = 5.5$^{\Delta}$	F (2, 24) = 2.6

Table 3.2 Ethological variables measured in DA and SD rats in the open-field.
Results shown as mean ± s.e.m., n = 9 in each group. DA rats were significantly different from SD rats, where *$p < 0.05$ and ***$p < 0.001$; responses were significantly different between test days, where $^{\Delta}p < 0.05$.

However, grooming behaviour in the SD rats increased on consecutive test days, resulting in a main effect of DAY, but no interaction. Freezing behaviour did not differ either between the two strains or on different days. Rearing behaviour also did not differ between the two strains, but increased in SD rats over the three test days and, in the DA group, decreased on Day 2 compared to Day 1, then increased on Day 3 compared to both previous days. This resulted in a main effect of DAY, but no interaction.

3.3.3 Behaviour of DA and SD rats in automated activity meters

Behaviour of the two strains in the automated activity meter illustrated clear differences between DA and SD rats. DA rats demonstrated significantly lower overall locomotor activity (Figure 3.4a), shown by reduced total numbers of infrared beam-breaks, resulting in a main effect of STRAIN. In addition, both DA and SD groups demonstrated a decrease in total activity over time, resulting in a main effect of TIME. However, there was no interaction between the effects of STRAIN and TIME. Rearing activity was also significantly lower in the DA rats and decreased over time in both strains (Figure 3.4b) resulting in main effects of both STRAIN and TIME but no interaction.

3.3.4 Effects of diazepam on the behaviour of DA and SD rats on the elevated plus-maze

In the next series of experiments, the effect of diazepam treatment on the behaviour of DA and SD rats was examined on the elevated plus-maze. With regard to anxiety-related behaviour observed in SD rats, diazepam treatment (1 mg/kg s.c.) led to an increased % number of open arm entries (Figure 3.5a) and increased % time spent on the open arms (Figure 3.5b). Diazepam treatment did not alter the % number of open arm entries performed by DA rats (Figure 3.5a), but did result in a greater % time being spent on the open arms by this strain (Figure 3.5b). Thus main effects of TREATMENT were observed with respect to both of these behavioural measures. However, there were no differences between strains with respect to open arm behaviour, either in diazepam-treated or vehicle-treated groups, thus neither main effects of STRAIN nor interactions were observed.

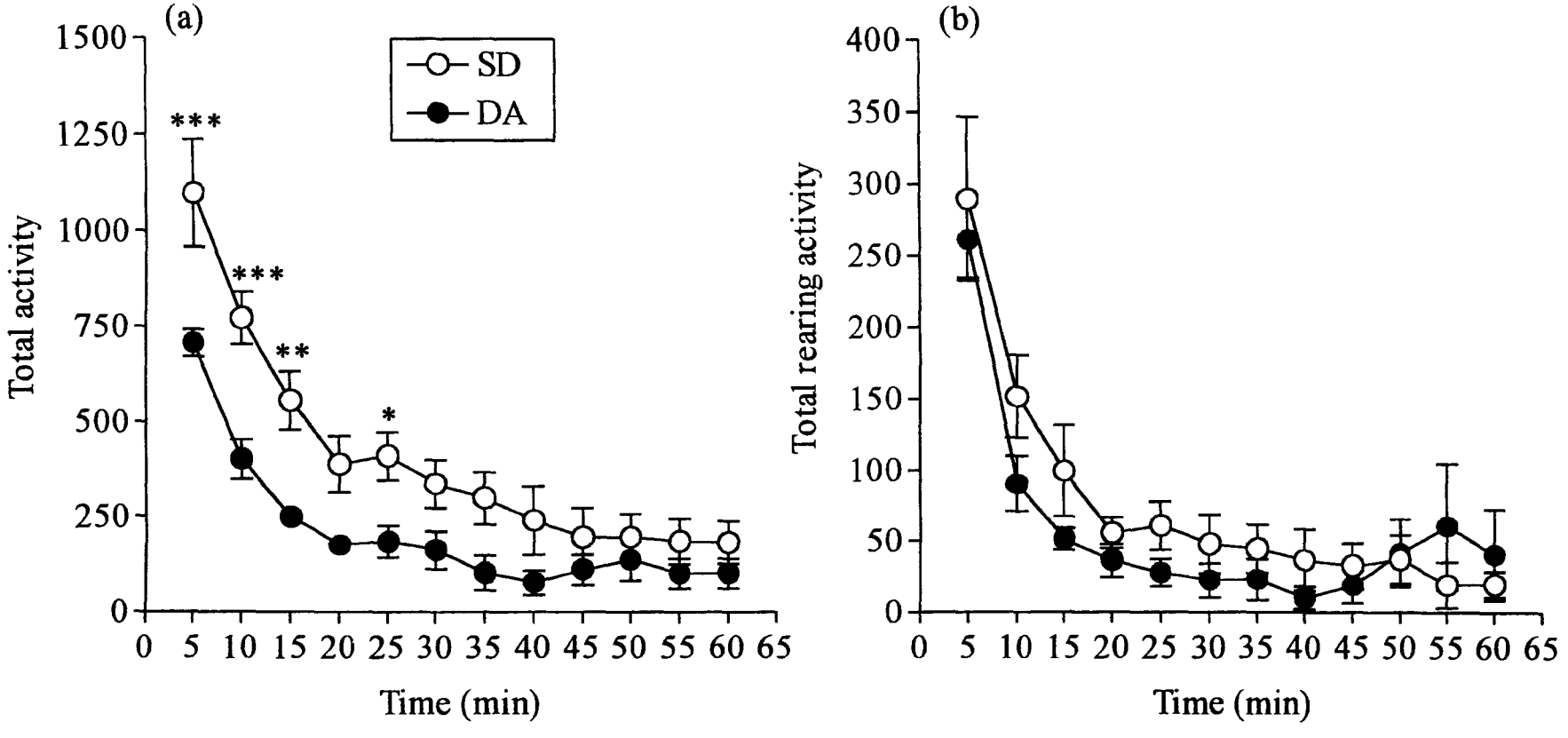

Figure 3.4 The behaviour of DA and SD rats in automated activity meters.

Results are shown as mean ± s.e.m., n = 8 in each group. ***(a)*** *Total activity.* DA rats were different from SD rats ($F(1, 84) = 78.8$, $p < 0.0001$), post-hoc analysis demonstrating statistical significance during the first 25 minutes (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). There was a decrease in activity over time ($F(11, 84) = 21.6$, $p < 0.0001$). ***(b)*** *Rearing activity.* DA rats were different from SD rats ($F(1, 84) = 7.5$, $p < 0.01$) and there was a decrease in activity over time ($F(11, 84) = 12.0$, $p < 0.0001$).

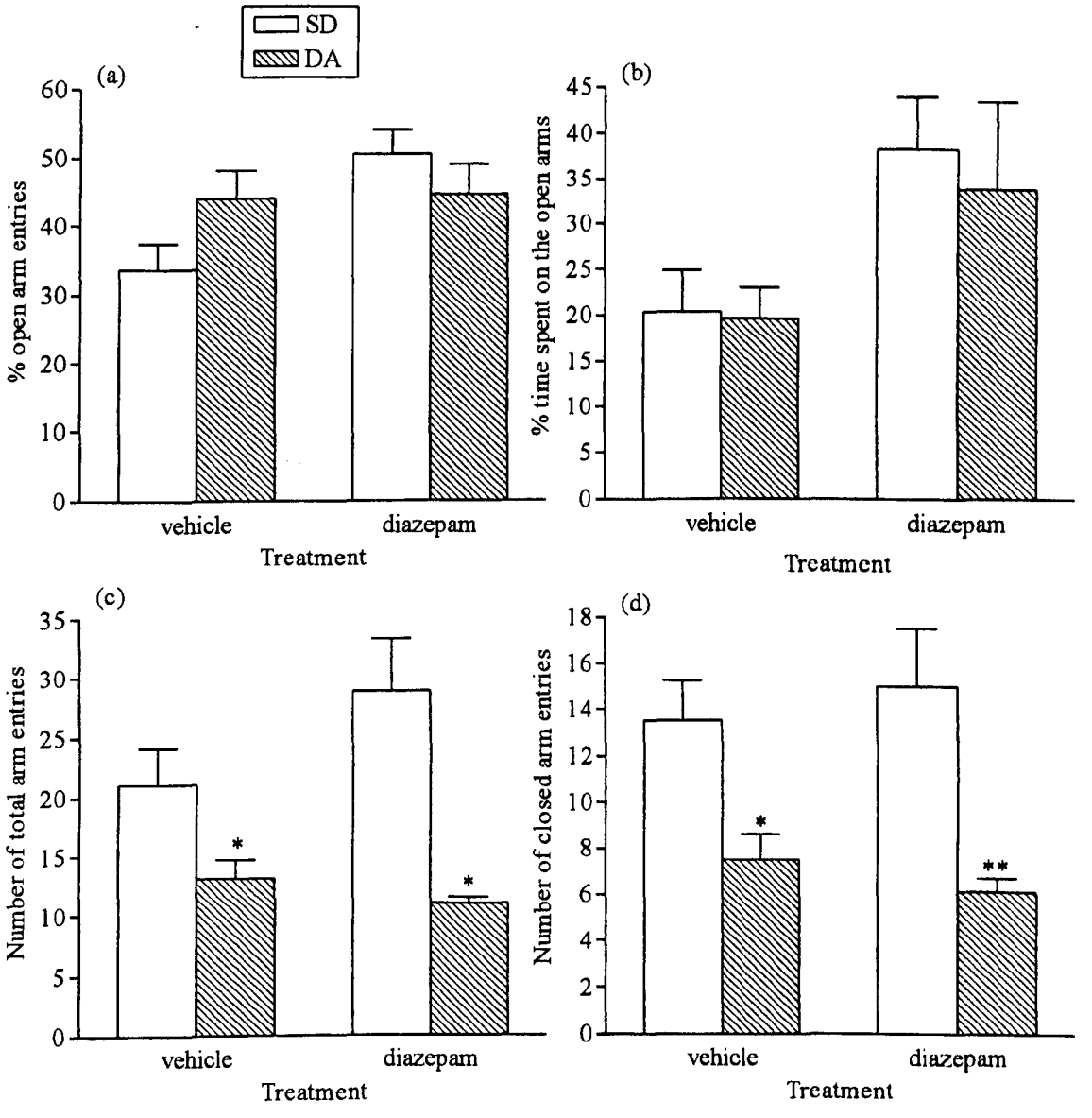

Figure 3.5 The effect of diazepam (1 mg/kg) on the behaviour of DA and SD rats on the elevated plus-maze.

Results shown as mean ± s.e.m., n = 8 in each group. ***(a)*** *Open arm entries, calculated as a % of total arm entries.* DA rats were not different from SD rats. Diazepam-treated rats were different from vehicle-treated rats ($F(1, 28) = 4.7$, $p < 0.05$). ***(b)*** *Time spent on open arms, calculated as a % of time spent on all arms.* DA rats were not different from SD rats. Diazepam-treated rats were different from vehicle-treated rats ($F(1, 28) = 6.7$, $p < 0.05$). ***(c)*** *Total arm entries.* DA rats were different from SD rats ($F(1, 28) = 21.3$, $p < 0.0001$) with post-hoc analysis showing a significant difference between strains in both diazepam-treated and vehicle-treated animals (*$p < 0.05$). There was no difference between diazepam-treated and vehicle-treated rats. ***(d)*** *Closed arm entries.* DA rats were different from SD rats ($F(1, 28) = 20.0$, $p < 0.001$) with post-hoc analysis showing a significant difference between strains in both diazepam-treated (**$p < 0.01$) and vehicle-treated (*$p < 0.05$) animals. There was no difference between diazepam-treated and vehicle-treated animals.

	Vehicle		Diazepam		Two-way ANOVA		
	SD	DA	SD	DA	STRAIN	TREATMENT	Interaction
Defecation	0.0 ± 0.0	1.8 ± 0.9	0.0 ± 0.0	3.4 ± 0.9**	F (1, 28) = 16.8***	F (1, 28) = 1.7	F (1, 28) = 1.7
Grooming	0.4 ± 0.3	4.5 ± 0.7***	0.6 ± 0.2	1.8 ± 0.6	F (1, 28) = 31.3***	F (1, 28) = 7.1$^{\Delta}$	F (1, 28) = 10.2§§
Head dips	21 ± 4.3	4.3 ± 0.9**	26.9 ± 4.1	5.3 ± 0.9***	F (1, 28) = 36.7***	F (1, 28) = 1.3	F (1, 28) = 0.6
Rearing	17.3 ± 1.7	24.8 ± 1.7	15 ± 3.3	18 ± 2.8	F (1, 28) = 4.5*	F (1, 28) = 3.3	F (1, 28) = 0.8
SAP	6.3 ± 1.2	4.1 ± 0.6	2.8 ± 0.9	3 ± 0.8	F (1, 28) = 1.1*	F (1, 28) = 6.5$^{\Delta}$	F (1, 28) = 1.7
Sniffing (directed)	38.4 ± 2.7	27.3 ± 4.9	44.1 ± 4.7	26.9 ± 2.3**	F (1, 28) = 13.9***	F (1, 28) = 0.5	F (1, 28) = 0.7
Sniffing (non-directed)	12.9 ± 2.6	14.8 ± 2	10 ± 2.3	11 ± 2.2	F (1, 28) = 0.4	F (1, 28) = 2.1	F (1, 28) = 0.0

Table 3.3 Ethological variables measured in DA and SD rats, pretreated with diazepam (1 mg/kg) or vehicle 30 min prior to being tested on the elevated plus-maze.

Results shown as mean ± s.e.m., n = 8 in each group. DA rats significantly different from SD rats, where **$p < 0.01$ and ***$p < 0.001$; diazepam-treated rats significantly different from vehicle-treated rats, where $^{\Delta}p < 0.05$; significant interaction between STRAIN and TREATMENT effects, where $^{\S\S}p < 0.01$.

Diazepam treatment had no effect on locomotor activity, within either strain, as both total (Figure 3.5c) and closed (Figure 3.5d) arm entries were unaltered compared to vehicle-treated groups. Thus main effects of TREATMENT were not observed. Diazepam- and vehicle-treated DA rats demonstrated significantly lower overall locomotor activity than both SD treatment groups, shown by fewer numbers of total (Figure 3.5c) and closed (Figure 3.5d) arm entries. This resulted in main effects of STRAIN with respect to both measures, while there were no interactions between the effects of TREATMENT and STRAIN in either behaviour.

With regard to the ethological variables measured in the elevated plus-maze (Table 3.3), diazepam treatment resulted in a significant decrease in grooming behaviour performed by DA rats and a significant decrease in stretched attend postures performed by SD rats, resulting in main effects of TREATMENT in both cases. DA rats demonstrated greater defecation, grooming and rearing behaviour compared to the SD strain, resulting in main effects of STRAIN in each case. Post-hoc analysis showed statistically significant differences in defecation in diazepam-treated rats ($p < 0.01$) and in grooming behaviour in vehicle-treated rats ($p < 0.001$). An interaction between STRAIN and TREATMENT effects was also observed with regard to grooming behaviour. DA rats performed significantly fewer head dips and significantly less directed sniffing than SD rats, resulting in main effects of STRAIN in both cases. Post-hoc analysis demonstrated statistically significant differences between strains with regard to head dips, in both diazepam-treated ($p < 0.001$) and vehicle-treated ($p < 0.01$) groups. With regard to directed sniffing, post-hoc analysis demonstrated statistical significance in diazepam-treated rats ($p < 0.01$). There was no difference within either strain with regard to either of these measures, therefore no main effects of TREATMENT or interactions were observed. There was no difference between strains or treatment groups with respect to non-directed sniffing.

Two weeks later, all animals which had previously been treated with diazepam were administered a second, higher dose of diazepam (1.5 mg/kg), while all animals which had previously been administered vehicle were again injected with vehicle, 30 min prior to being tested on the elevated plus-maze.

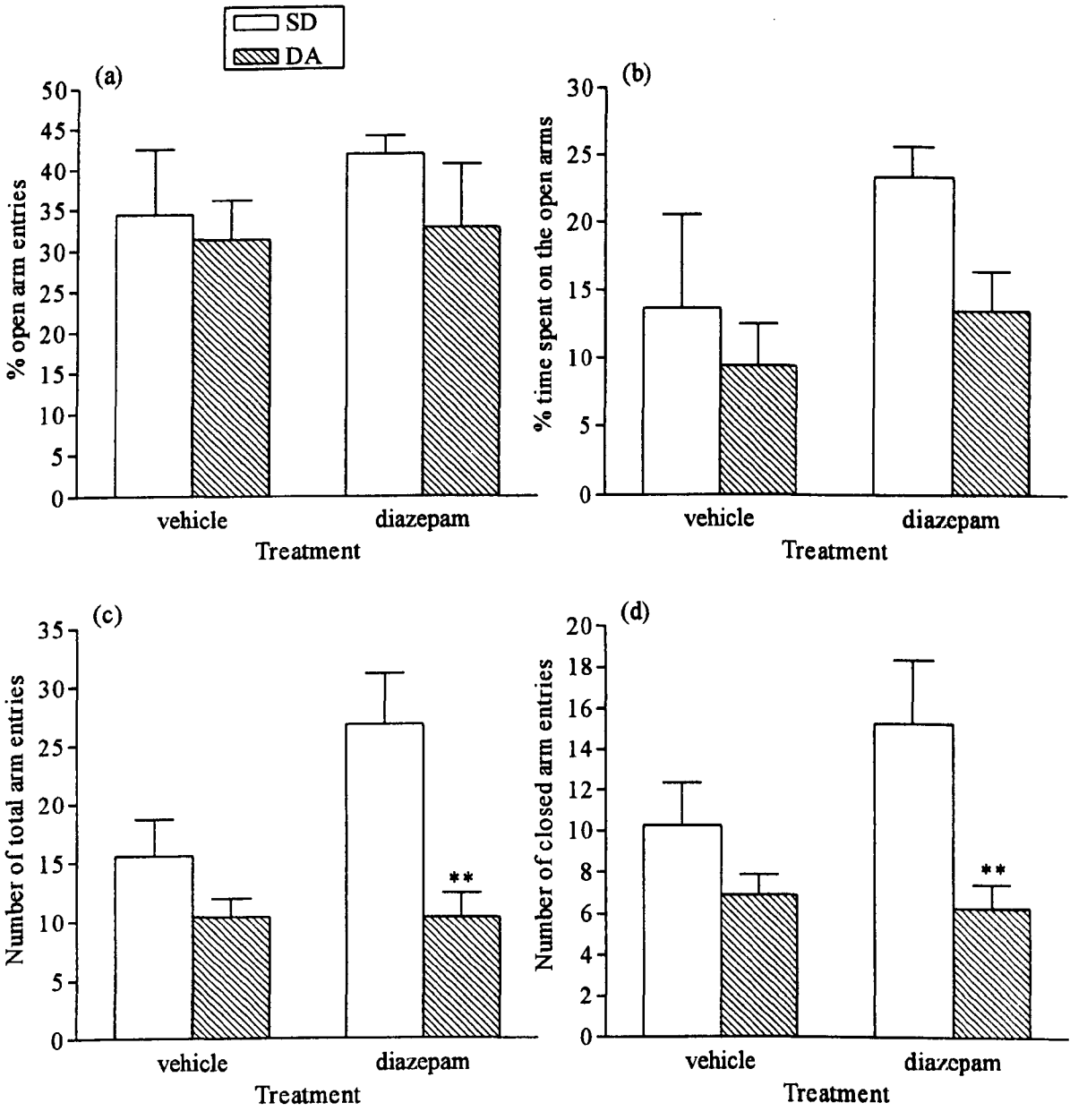

Figure 3.6 The effect of diazepam (1.5 mg/kg) on the behaviour of DA and SD rats on the elevated plus-maze.

Results shown as mean ± s.e.m., n = 8 in each group. ***(a)*** *Open arm entries, calculated as a % of total arm entries.* There were no significant differences either between strains or between treatment groups. ***(b)*** *Time spent on open arms, calculated as a % of time spent on all arms.* There were no significant differences either between strains or between treatment groups. ***(c)*** *Total arm entries.* DA rats were different from SD rats ($F(1, 27) = 14.2$, $p < 0.001$), with post-hoc analysis showing a significant difference between strains in diazepam-treated animals (**$p < 0.01$). There was no difference between diazepam-treated and vehicle-treated rats. ***(d)*** *Closed arm entries.* DA rats were different from SD rats ($F(1, 27) = 10.3$, $p < 0.01$), with post-hoc analysis showing a significant difference between strains in diazepam-treated animals (**$p < 0.01$). There was no difference between diazepam-treated and vehicle-treated rats.

	Vehicle		Diazepam		Two-way ANOVA		
	SD	DA	SD	DA	STRAIN	TREATMENT	Interaction
Defecation	0.1 ± 0.1	2.8 ± 0.9**	0.0 ± 0.0	2.1 ± 0.5*	F (1, 28) = 21.3***	F (1, 28) = 0.5	F (1, 28) = 0.2
Grooming	1.1 ± 0.4	5.6 ± 0.8***	1.3 ± 0.2	2.6 ± 0.7	F (1, 28) = 25.4***	F (1, 28) = 6.1$^{\Delta}$	F (1, 28) = 7.2§
Head dips	7.9 ± 2.6	1.5 ± 0.7	20.4 ± 4.1	3.5 ± 1.5***	F (1, 28) = 20.3***	F (1, 28) = 7.9$^{\Delta\Delta}$	F (1, 28) = 4.1
Rearing	11.0 ± 3.2	23.1 ± 2.9*	16.4 ± 3.0	18.8 ± 3.7	F (1, 28) = 5.1*	F (1, 28) = 0.0	F (1, 28) = 2.3
SAP	3.1 ± 0.9	3.3 ± 1.2	3.9 ± 1.0	2.5 ± 0.5	F (1, 28) = 0.4	F (1, 28) = 0.0	F (1, 28) = 0.6
Sniffing (directed)	29.6 ± 3.3	25.9 ± 3.0	40.0 ± 3.7	21.6 ± 2.1***	F (1, 28) = 12.7**	F (1, 28) = 1.0	F (1, 28) = 5.5§
Sniffing (non-directed)	14.6 ± 3.2	18.6 ± 2.9	11.4 ± 2.8	16.3 ± 2.4	F (1, 28) = 2.5	F (1, 28) = 1.0	F (1, 28) = 0.0

Table 3.4 Ethological variables measured in DA and SD rats, pretreated with diazepam (1.5 mg/kg) or vehicle 30 min prior to being tested on the elevated plus-maze.

Results shown as mean ± s.e.m., n = 8 in each group. DA rats significantly different from SD rats, where *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; diazepam-treated rats significantly different from vehicle-treated rats, where $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$; significant interaction between STRAIN and TREATMENT effects, where $^{\S}p < 0.05$.

With regard to anxiety-related behaviour, there were no differences between strains or between treatment groups in either the % number of open arm entries (Figure 3.6a) or the % time spent on the open arms (Figure 3.6b), thus no main effects of STRAIN, TREATMENT or interaction were observed. With regard to locomotor activity, diazepam treatment had no effect on the number of total (Figure 3.6c) or closed (Figure 3.6d) arm entries, thus there was no main effect of TREATMENT. Diazepam-treated DA rats performed fewer total (Figure 3.6c) and closed (Figure 3.6d) arm entries than diazepam-treated SD rats, resulting in main effects of STRAIN in both cases. There were no differences in either of these measures between vehicle-treated DA and SD rats.

With regard to the ethological variables measured (Table 3.4), the differences between strains were similar to those seen in the previous experiment (compare with Table 3.3). Diazepam treatment resulted in a significant decrease in grooming behaviour performed by DA rats and a significant increase in head dips performed by SD rats, resulting in main effects of TREATMENT in both cases. DA rats demonstrated greater defecation, grooming and rearing behaviour, compared to SD rats, resulting in main effects of STRAIN. Post-hoc analysis showed statistically significant differences in defecation between strains in both diazepam-treated ($p < 0.05$) and vehicle-treated ($p < 0.01$) groups. Post-hoc statistical significance was also demonstrated in the grooming ($p < 0.001$) and rearing ($p < 0.05$) behaviour of vehicle-treated groups and an interaction between STRAIN and TREATMENT was observed with regard to grooming behaviour. DA rats performed fewer head dips and less directed sniffing than SD rats, resulting in main effects of STRAIN in both cases. Post-hoc analysis demonstrated statistically significant differences between strains in diazepam-treated rats, with regard to both of these behaviours ($p < 0.001$). SAP and non-directed sniffing behaviours did not differ either between strains or between treatment groups.

3.4 DISCUSSION

This study revealed pronounced differences in the behaviour of naïve DA and SD rats. DA rats showed a higher level of anxiety-related behaviour and lower locomotor

activity than the SD strain. Furthermore, these differences were seen to be consistent across the three different behavioural paradigms employed.

On the elevated plus maze DA rats demonstrated higher anxiety-related behaviour, clearly illustrated by performing a lower % number of open arm entries and by spending less % time on the open arms, compared to SD rats. In addition, the DA rats displayed lower locomotor activity, as shown by fewer total and closed arm entries. It should be noted that measurement of closed arm entries provides a better indication of locomotor activity, as it is unaffected by anxiety-related measures such as open arm entries (Cruz *et al.*, 1994). These differences in behaviour between the two strains were apparent both on Day 1 and again when the animals were tested 25 days later. These data are also consistent with the observations of King (1999) who found that DA rats performed fewer open arm entries, spent less time on the open arms and demonstrated lower overall locomotor activity than both SD and HL rats.

There were no statistically significant differences in the anxiety-related and locomotor behaviour of either strain between the two test days. As discussed in section 3.1.4, repeated exposure of rats to the elevated plus-maze has been reported to alter the type of "anxiety" which is being measured and, in particular, alters the response to benzodiazepines in later trials (File *et al.*, 1990). Where "one-trial tolerance" has been demonstrated, there tends to be a far shorter interval between trials than that employed in the current study. For example, tolerance to the anxiolytic effect of chlordiazepoxide, as demonstrated by File *et al.* (1990), involved an inter-trial interval of 24 h or a maximum of two weeks. In addition, habituation effects are generally not reported in control animals (Pellow *et al.*, 1985; File *et al.*, 1990), therefore a 25 day inter-trial interval was deemed sufficient for the current study. Furthermore, by increasing the elevated plus-maze trial from 5 min to 10 min, "one-trial tolerance" to the anxiolytic effects of chlordiazepoxide can be reversed (File *et al.* 1990). Therefore, the 10 min plus-maze trial employed here should have further served to minimise any potential effects of re-testing.

Similar differences in anxiety-like behaviour were observed in the open-field, where DA rats performed far fewer Zone 2 crossings, perhaps indicating less "risk-taking" behaviour. By remaining in Zone 1, close to the high surrounding wall of the open-field, the DA rats avoided the open space of the centre of the arena, which signified the conflict between an exploratory drive and avoidance of open, brightly lit spaces (see Schmitt & Hiemke, 1998a). Locomotor behaviour was also lower in the DA rats, shown by fewer total numbers of crossings compared to the SD rats. The first two days of testing in the open-field were performed under white light illumination in order to test the effects of habituation, while the third day was performed under red light illumination, which it was hoped would provide a less anxiogenic environment. Although the number of Zone 2 crossings performed by SD rats increased over the three test days, there was no significant difference between days. There was also no difference between days within either strain with respect to the total number of crossings. Therefore, there was no apparent habituation effect and no effect of illumination.

The differences in behaviour between the two strains were further exemplified in the automated activity meter. DA rats demonstrated lower locomotor activity, compared to SD rats, as shown by significantly fewer total numbers of infrared beam breaks.

Several of the ethological variables measured also served to reinforce the differences between the two strains. Defecation was significantly higher in DA rats, on both the elevated plus-maze and in the open field, a response which is consistent with increased "anxiety" or "emotionality" (Hall, 1934b). Grooming behaviour was also significantly higher in the DA rats, being termed a "displacement behaviour" in conflict situations (see Cruz *et al.*, 1994 and Pellow *et al.*, 1985), and could therefore be expected to increase in more "anxious" animals. On the elevated plus-maze, DA rats performed significantly fewer head dips than SD rats. This behaviour is an indicator of low levels of anxiety-related behaviour, being increased by anxiolytic drugs (Cruz *et al.*, 1994; Griebel *et al.*, 1997b), and thus serves as further proof of greater anxiety-related behaviour in DA rats.

DA rats performed greater rearing behaviour on the elevated plus-maze (particularly on Day 26), but less rearing behaviour in the automated activity meter, compared to SD rats. This behaviour is believed to reflect locomotor activity, being a variable which loads heavily and exclusively on the activity factor during ethopharmacological behavioural analysis (Cruz *et al.*, 1994; File, 1992; Rodgers & Dalvi, 1997). However, although increased rearing on the plus-maze may not be consistent with the other measures of locomotor activity in this study, rearing may also be an indicator of anxiety-related behaviour. Rodgers & Dalvi (1997) reported a decrease in rearing behaviour of mice tested on the elevated plus-maze in response to muscimol, a $GABA_A$ receptor agonist. Muscimol did not alter locomotor activity, shown by total and closed arm entries, but did significantly increase % time spent on the open arms, in addition to significantly decreasing rearing behaviour. The authors suggested that muscimol had anti-anxiety effects which are behaviourally selective and that the reduction in rearing was a "consequence of anxiolysis" (Rodgers & Dalvi, 1997).

There was no significant difference between the two strains with regard to stretched attend posture, which is considered to be an anxiety-related (risk assessment) behaviour (Cruz *et al.*, 1994; Griebel *et al.*, 1997b; Rodgers & Dalvi, 1997). Risk assessment is reported to decrease in response to anxiolytic drugs (Griebel *et al.* 1997b) and may therefore be expected to be higher in more "anxious" animals. Although there was no difference between the two strains with respect to directed sniffing, DA rats performed significantly greater non-directed sniffing, which may also be considered to be a risk assessment behaviour (see Rodgers & Dalvi, 1997) and is, therefore, consistent with the majority of other ethological measures reported here.

The pronounced differences in behaviour between the two strains led to the suggestion that they may also differ in their responses to pharmacological manipulation. Thus, a separate group of animals were administered diazepam, an anxiolytic compound which has frequently been used when testing animals on the elevated plus-maze (e.g. Brett & Pratt, 1990; File *et al.*, 1993a; Handley & Mithani, 1984; Pellow *et al.*, 1985; Treit *et al.*, 1993) and has been shown to increase both open arm exploration (e.g. Handley &

Mithani, 1984; Pellow *et al.*, 1985; Treit *et al.*, 1993; Wright *et al.*, 1992) and the time spent on the ends of the open arms (Wright *et al.*, 1992).

Following administration of the lower dose of diazepam (1 mg/kg), a clear anxiolytic response was observed in SD rats, shown by a significant increase in the % number of open arm entries and the % time spent on the open arms, and a non-significant increase in locomotor activity. The behavioural effects of diazepam in DA rats were less clear-cut. There was no difference in the % number of open arm entries between diazepam- and vehicle-treated DA rats, although diazepam treatment did result in an increased % time spent on the open arms indicating an anxiolytic effect. However, neither DA treatment group was significantly different from the SD rats with regard to open arm behaviour. Thus, the marked differences observed in naïve animals were not seen where animals were injected with vehicle, with respect to anxiety-related measures. It appears that brief handling, whilst injecting vehicle, abolishes the high levels of "anxiety" seen in naïve DA rats. In fact the % number of open arm entries and the % time spent on the open arms by vehicle-treated DA rats were approximately three-fold higher than the values recorded in naïve DA rats (compare Figures 3.5a and 3.5b with Figures 3.2a and 3.2b). However, such differences did not extend to locomotor behaviour or ethological variables, the marked differences between strains being similar to those seen in naïve rats; significant differences in locomotor behaviour between strains were still observed, shown by fewer total and closed arm entries in the DA rats. Locomotor activity was not significantly changed by diazepam in either strain, indicating that this dose of diazepam did not produce sedation. Both diazepam- and vehicle-treated DA rats performed greater defecation and grooming behaviour than SD rats, as seen in naïve animals. Diazepam administration had no effect on defecation behaviour but did reduce grooming behaviour in DA rats, which is consistent with an anxiolytic effect.

As discussed in section 3.1.4, anxiety-like behaviour can be attenuated by handling and/or repeated testing on the elevated plus-maze. For example, Andrews & File (1993) demonstrated a significant reduction in open arm behaviour in HL rats which had not been handled, compared to those which had been handled daily for seven days. This is consistent with the effects seen in vehicle-treated DA rats in this study, which

demonstrated an increase in open arm behaviour compared to naïve rats. Brett & Pratt (1990) demonstrated that a chronic handling and injecting regimen abolished the anxiolytic effects of diazepam. This result is consistent with the % number of open arm entries in the current study which did not differ between vehicle- and diazepam-treated DA rats, although the % time spent on the open arms was significantly increased in diazepam-treated DA rats, compared to control animals. The differences between naïve and vehicle-treated DA rats are made more remarkable by the fact that the handling procedure was acute, being only during the injection procedure. Previous studies have not compared naïve with treated animals, as even unhandled animals have been injected with vehicle. However, handling, to whatever extent, has still been shown to alter subsequent behavioural responses on the elevated plus-maze (Andrews & File, 1993; Brett & Pratt, 1990).

During the second diazepam experiment, where a higher dose (1.5 mg/kg) was administered two weeks after the first dose, open arm behaviour and locomotor behaviour were modestly reduced in all groups, compared to the first diazepam experiment (compare Figure 3.6 with Figure 3.5). This may have been due to habituation, although this seems unlikely based on the results seen in naïve animals, and the effects of diazepam (in addition to any differences between strains) were still apparent at the higher dose.

In conclusion, naïve DA rats display significantly higher anxiety-like behaviour than SD rats, which was seen to be consistent across the different behavioural paradigms employed in the current study. These differences were attenuated by acute handling and vehicle injection, which made it difficult to accurately assess any anxiolytic effect of diazepam in DA rats on the elevated plus-maze. This indicates that DA rats may be unsuitable for testing the effects of putative anxiolytic or anxiogenic compounds. However, although the magnitude of responses of naïve DA rats were markedly different from SD rats, anxiety-related and locomotor behaviours were qualitatively similar, indicating that DA rats are suitable for investigating the longer-term effects of MDMA administration using these behavioural paradigms.

CHAPTER 4

BEHAVIOURAL TESTING II:

A STUDY OF THE EFFECTS OF MDMA ADMINISTRATION ON THE LONG-TERM BEHAVIOUR OF DARK AGOUTI RATS IN THE ELEVATED PLUS-MAZE AND OPEN FIELD

4 BEHAVIOURAL TESTING II: A study of the effects of MDMA administration on the long-term behaviour of Dark Agouti rats in the elevated plus-maze and open field

4.1 INTRODUCTION

Alterations in serotonergic activity induced by neurotoxins such as 5,7-DHT or PCPA result in reduced anxiety-like behaviour in the elevated plus-maze (Briley *et al.*, 1990; Hall *et al.*, 1999), punished responding tests (Tye *et al.*, 1977; 1979) and social interaction tests (File & Hyde, 1977) (see section 3.1.6). Therefore, the depletion of 5-HT which results from MDMA administration to rats (e.g. Colado *et al.*, 1993; Commins *et al.*, 1987; O'Hearn *et al.*, 1988; Schmidt, 1987a) might be expected to have a similar effect.

The majority of animal studies investigating the behavioural effects of MDMA have focused on the acute phase of action of the drug, which involves both increased locomotor behaviour, characteristic of dopaminergic stimulation (see Callaway *et al.*, 1990; Dafters, 1994; Kehne *et al.*, 1996a; McCreary *et al.*, 1999; McNamara *et al.*, 1995; O'Loinsigh *et al.*, 2001), and a behavioural syndrome, characteristic of serotonergic activation (see Colado *et al.*, 1993; De Souza *et al.*, 1997; Marston *et al.*, 1999; Shankaran & Gudelsky, 1999; Slikker Jr. *et al.*, 1989; Spanos & Yamamoto, 1989). The current chapter is concerned with the longer-term effects of a single dose of MDMA on behaviour, as measured in two commonly used animal models of "anxiety": the elevated plus-maze and open-field. The effects of MDMA and other amphetamine-derived compounds on the acute and long-term responses of rats and mice in both behavioural paradigms are discussed in the current chapter, while utilisation of the plus-maze and open-field as behavioural models has been discussed in Chapter 3.

4.1.1 Effects of amphetamine derivatives on behaviour on the elevated plus-maze

4.1.1.1 Acute effects

Lin *et al.* (1999) compared the responses of male Quackenbush Swiss mice on the elevated plus-maze following acute MDMA and D-amphetamine administration (30 min prior to testing). MDMA induced dose-dependent effects on anxiety-related behaviour, being anxiogenic at lower doses and anxiolytic at higher doses. At a dose of 1 mg/kg, there was no effect on the % time spent on the open arms, % number of open arm entries or enclosed arm entries; at 4 mg/kg, the % number of open arm entries was significantly decreased while numbers of enclosed arm entries increased, the latter indicating a hyperactive effect; at 12 mg/kg, no significant differences were seen in any of the behavioural measures; at 20 mg/kg, a significant increase in the % time spent on the open arms was observed. These responses were in contrast to the effects of amphetamine, which induced a dose-dependent anxiogenic effect, shown by a decrease in the % time spent on the open arms (Lin *et al.*, 1999).

Morley & McGregor (2000) tested male Wistar rats on the elevated plus-maze 25 min after administration of MDMA (1.25, 2.5 or 5 mg/kg) and demonstrated a dose-related decrease in the time spent on the open arms and the total number of arm entries. Although these results indicated an anxiogenic effect, the authors suggested that changes in locomotor behaviour could have some bearing on the changes seen in anxiety-like behaviour (Morley & McGregor, 2000). Furthermore, subjects were tested on the elevated plus-maze immediately after an emergence test (rats were placed in a black box in the corner of an open field and the latency to emerge and open field activity were measured), which may have affected anxiety-related behaviour on the plus-maze.

4.1.1.2 Longer-term effects

Olausson *et al.* (2000) administered amphetamine (1 mg/kg s.c.) to male SD rats, daily for 15 days. One group of rats were also chronically administered citalopram (5 mg/kg s.c., 60 min prior to each amphetamine dose, daily for 15 days). Behaviour on the elevated plus-maze was monitored for a 5 min test period on Day 17. Chronic

amphetamine treatment resulted in a significant increase in the % time spent on the open arms of the plus-maze compared to both control rats and acutely treated animals (those which were tested 35 - 40 min after a single 1 mg/kg dose of amphetamine). While the % number of open arm entries and total number of arm entries were also significantly increased in chronically-treated animals compared to control animals, there was no difference between chronic and acute amphetamine treatment with regard to these measures. Chronic citalopram pretreatment significantly attenuated the amphetamine-induced increase in % time spent on the open arms, but had no effect on the % number of open arm entries. Acute administration of 5-hydroxytryptophan + benserazide (decarboxylase inhibitor) to chronic amphetamine-treated animals abolished the amphetamine-induced increases in % time spent on the open arms, % number of open arm entries and total arm entries on the plus-maze. The authors concluded that repeated amphetamine treatment had a disinhibitory effect on elevated plus-maze behaviour (shown by increasing the % time spent on the open arms). The attenuating effects of citalopram and 5-hydroxytryptophan were thought likely to involve increased serotonergic neurotransmission, which indicated 5-HT-, rather than dopamine-, mediated effects of amphetamine with regard to this behavioural model (Olausson *et al.*, 2000).

In contrast, Cancela *et al.* (2001) demonstrated an anxiogenic effect of chronic amphetamine treatment. Male Wistar rats were tested on the elevated plus-maze four days after the cessation of treatment, which comprised daily injections (2 mg/kg) for nine consecutive days. Amphetamine-treated rats spent a significantly smaller % time on the open arms, an effect which was abolished by pretreatment with either the non-selective dopamine antagonist haloperidol, or the selective dopamine D_1 receptor antagonist SCH 23390. These results indicated that dopamine D_1 receptors play an important role in the chronic amphetamine-induced changes in behaviour, measured on the elevated plus-maze (Cancela *et al.*, 2001). However, since neither Olausson *et al.* (2000) nor Cancela *et al.* (2001) reported analysis of brain monoamine concentrations, it cannot be determined whether the observed behavioural changes were due to neurotoxic effects of chronic amphetamine treatment, or simply effects of chronic dosing.

4.1.2 Effects of amphetamine derivatives on behaviour in the open field

4.1.2.1 Acute effects

McCreary *et al.* (1999) investigated the behaviour of male SD rats in an open-field/activity meter apparatus, comprising a 40 cm^3 arena with two rows of infrared beams located 4 and 16 cm above the base of the apparatus. General locomotor activity, central activity (movement within the inner 16 cm^2 area) and rearing activity, were assessed by monitoring the numbers of lower and upper level infra-red beam breaks during each test period. MDMA (3 mg/kg s.c.) was administered immediately prior to testing which consisted of 5 min test periods for a total of 90 min. MDMA induced a hyperactivity response, shown by increased activity in both the peripheral and central areas of the apparatus, while rearing activity was not consistently altered. Pretreatment with the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 blocked the MDMA-induced hyperactivity, while the 5-HT_{1A} antagonist WAY 100635 had no effect on the hyperactivity response (McCreary *et al.*, 1999). GR 127935 has been shown to block 5-HT-mediated dopamine efflux in the rat prefrontal cortex (Iyer & Bradberry, 1996), and therefore may have blocked MDMA-induced hyperactivity by preventing 5-$HT_{1B/1D}$ mediation of dopamine efflux (McCreary *et al.*, 1999).

O'Loinsigh *et al.* (2001) tested the acute behavioural effects of MDMA, MDEA and MDBA on male Wistar rats, which had been acclimatised to the open field apparatus for two days (5 min exposure per day) prior to drug treatment. Rats were administered the amphetamine twice daily for four consecutive days (20 mg/kg dose) and tested 30 min after the first injection on each day. MDMA administration resulted in a pronounced increase in locomotor activity on each of the four treatment days, compared to all other treatment groups. MDEA also induced a significant increase in locomotor activity, compared to control and MDBA groups, while MDBA had no effect on locomotion. MDMA and MDEA significantly reduced rearing behaviour and induced stereotypy, Straub tail and head weaving, while MDBA had no effect on any of these behaviours (O'Loinsigh *et al.*, 2001). Morley & McGregor (2000) tested male Wistar rats in an open field (emergence) apparatus 20 min after administration of MDMA (1.25, 2.5 or 5 mg/kg), and demonstrated a significant increase in the latency to emerge from the black box, a significant decrease in the amount of time spent in the open field and an increase

in defecation behaviour. These results indicated an anxiogenic effect of acute MDMA treatment and were consistent with the results seen in the elevated plus-maze (Morley & McGregor, 2000).

4.1.2.2 Longer-term effects

McCreary *et al.* (1999) investigated the effect of chronic MDMA treatment (3 mg/kg s.c. once per day for five consecutive days) on behaviour in the open-field/activity meter apparatus. A significant increase in central activity was observed in MDMA-treated animals on Day 5 compared to Day 1, indicating a sensitisation of this type of behaviour, while there was little difference in peripheral activity between the two days. Although GR 127935 blocked both peripheral and central activity on Day 1, it had no effect on MDMA-induced hyperactivity within either region on Day 5. The authors suggested that either the 5-$HT_{1B/1D}$ receptor subtypes have a minimal role in the development of behavioural sensitisation to MDMA or, with repeated administration of MDMA, the balance between 5-$HT_{1B/1D}$ and dopaminergic systems shifts towards the latter (McCreary *et al.*, 1999).

McNamara *et al.* (1995) investigated the behaviour of MDMA-treated male SD rats in the open-field and monitored their home-cage locomotor activity. The latter was monitored constantly for a total of eight days: (1) 24 h before drug treatment commenced; (2) four drug treatment days where rats were administered MDMA twice daily (5, 10 or 20 mg/kg in each case); and (3) three drug-free days. Animals were tested in the open-field on the final day of the experimental period, being monitored for the total number of crossings performed within 3 min. Administration of the two higher doses of MDMA resulted in a significant increase in total locomotor activity during the drug-treatment period, while levels had returned to control values 24 h after the last MDMA injection. There were no significant differences between MDMA-pretreated and control animals with regard to their behaviour in the open-field, three days after the last dose of MDMA. These results indicate that MDMA-induced changes in locomotor activity are dose- and time-dependent, while any behavioural effects are not apparent after cessation of drug treatment (McNamara *et al.*, 1995).

4.1.3 Aims of the investigation

There is little data available concerning the longer-term behavioural consequences of a neurotoxic dose of MDMA in rats, thus two simple models of anxiety-related behaviour were used for this purpose. The DA strain of rat has been used during the investigation of the effects of MDMA on rat body temperature and brain biochemistry (see Chapters 5 and 6), thus the suitability of this strain for behavioural testing was first assessed by comparing the anxiety-related and locomotor behaviour of DA rats with the responses of SD rats (see Chapter 3).

Once a behavioural baseline had been determined in DA rats, it was then possible to investigate the longer-term effects of MDMA administration on anxiety-related behaviour in this strain. Rats were administered a single neurotoxic dose of MDMA (12.5 mg/kg i.p.) or saline and their responses on the elevated plus-maze and in the open-field were assessed at three different time-points over a period of 80 days: (1) Days 8 - 11, one week after MDMA administration, at which time 5-HT content in rat brain is already markedly decreased (see Colado *et al.*, 1995; O'Shea *et al.*, 1998), and therefore any behavioural consequences may be expected to begin to appear; (2) Days 29 - 32, approximately one month after MDMA administration, which was believed to enable a suitable time period to elapse to prevent any habituation effects (see Chapter 3); and (3) Days 71 - 73 (open-field) and Day 80 (plus-maze), approximately 11 weeks after MDMA administration, at which time neurodegenerative effects should still be present prior to the onset of serotonergic recovery, which has been demonstrated 16 weeks after drug administration (Scanzello *et al.*, 1993).

4.2 METHODS

4.2.1 Animals and drug administration

Adult male DA rats were housed as detailed in section 2.1(2). MDMA was dissolved in 0.9 % NaCl w/v and injected at a dose of 12.5 mg/kg i.p. on a single occasion. Rectal temperature was monitored for 3.5 h following drug administration to provide an indication of *in vivo* efficacy of the dose of MDMA administered.

4.2.2 Behavioural assessment

DA rats were administered MDMA (12.5 mg/kg i.p.) or saline (n = 8 in each group) and tested on the elevated plus-maze in randomised order on three separate occasions: Day 8, Day 29 and Day 80. Animals were monitored for numbers of arm entries, time spent on open arms and several simple behaviours, as detailed in section 2.6.1.

All rats were also tested in the open-field on three separate occasions, on three consecutive days in each case: Days 9 – 11, Days 30 – 32 and Days 71 – 73. The first two days of each testing period were performed under white-light illumination and the third day under red-light illumination. Animals were monitored for numbers of zone crossings and anxiety-related behaviours (see section 2.6.2).

4.2.3 Statistics

Data was analysed by two-way ANOVA with repeated measures, with TREATMENT (MDMA or saline) as the between subject factor and DAY (day of testing) as the repeated measure, followed by Bonferroni post-hoc tests where appropriate.

4.3 RESULTS

4.3.1 Acute rectal temperature response to MDMA administration

MDMA administration resulted in an acute hyperthermic response, constituting a rapid rise in rectal temperature of 1.5 - 2 °C, which reached a peak 40 - 60 min post-injection and was sustained for over 3.5 h (see Figure 5.1, Chapter 5).

4.3.2 Long-term effects of MDMA on the behaviour of DA rats on the elevated plus-maze

MDMA-pretreated rats demonstrated differences in their behaviour on the elevated plus-maze compared to saline-treated control rats, which became apparent at the later time-points. One week after a single MDMA or saline injection (Day 8), the treatment groups did not differ with respect to anxiety-related behaviours, shown by % number of open arm entries (Figure 4.1a) and % time spent on the open arms (Figure 4.1b). Three

weeks later (Day 29), there were still no statistically significant differences between treatment groups with respect to either of the anxiety-related behaviours, although MDMA-pretreated animals demonstrated a marginal increase in both measures compared to control animals. On Day 80, MDMA-pretreated animals performed a 48 % greater % number of open arm entries compared to control animals, although this did not reach statistical significance (Figure 4.1a). In addition, MDMA-pretreated rats spent a significantly greater % time on the open arms on Day 80 compared to saline-pretreated rats (Figure 4.1b), resulting in a main effect of TREATMENT, with post-hoc analysis highlighting the difference between treatment groups on Day 80 ($p < 0.05$). Both treatment groups performed a lower % number of open arm entries and spent a lower % time on the open arms on Day 29 compared to Days 8 and 80, resulting in a main effect of DAY with respect to % time spent on the open arms. No interaction effect was observed in either of the anxiety-related behaviours measured.

With respect to locomotor activity, MDMA-pretreated rats performed greater numbers of both total (Figure 4.1c) and closed (Figure 4.1d) arm entries, resulting in significant main effects of TREATMENT in both cases. In particular, post-hoc analysis demonstrated a statistically significant difference in the number of total arm entries between treatment groups on Day 80 ($p < 0.05$). Both treatment groups performed fewer total and closed arm entries on Day 29 compared to the other two test days, resulting in significant main effects of DAY, but no significant interactions. With regard to the ethological variables measured in the elevated plus-maze (Table 4.1), there were no differences between MDMA-pretreated and control animals, thus no main effects of TREATMENT were observed. However, several of the behaviours measured were performed to a greater or lesser extent on different test days, resulting in main effects of DAY. For example, both treatment groups defecated more on Day 29 compared to Days 8 and 80 ($p < 0.05$), and both treatment groups performed greater numbers of head dips on Day 80 compared to the previous two test days ($p < 0.001$). Both groups performed greater numbers of stretched attend postures ($p < 0.0001$) and greater directed sniffing on Day 8 compared to the latter test days ($p < 0.0001$), while there were no differences between days with respect to both rearing behaviour and non-directed sniffing.

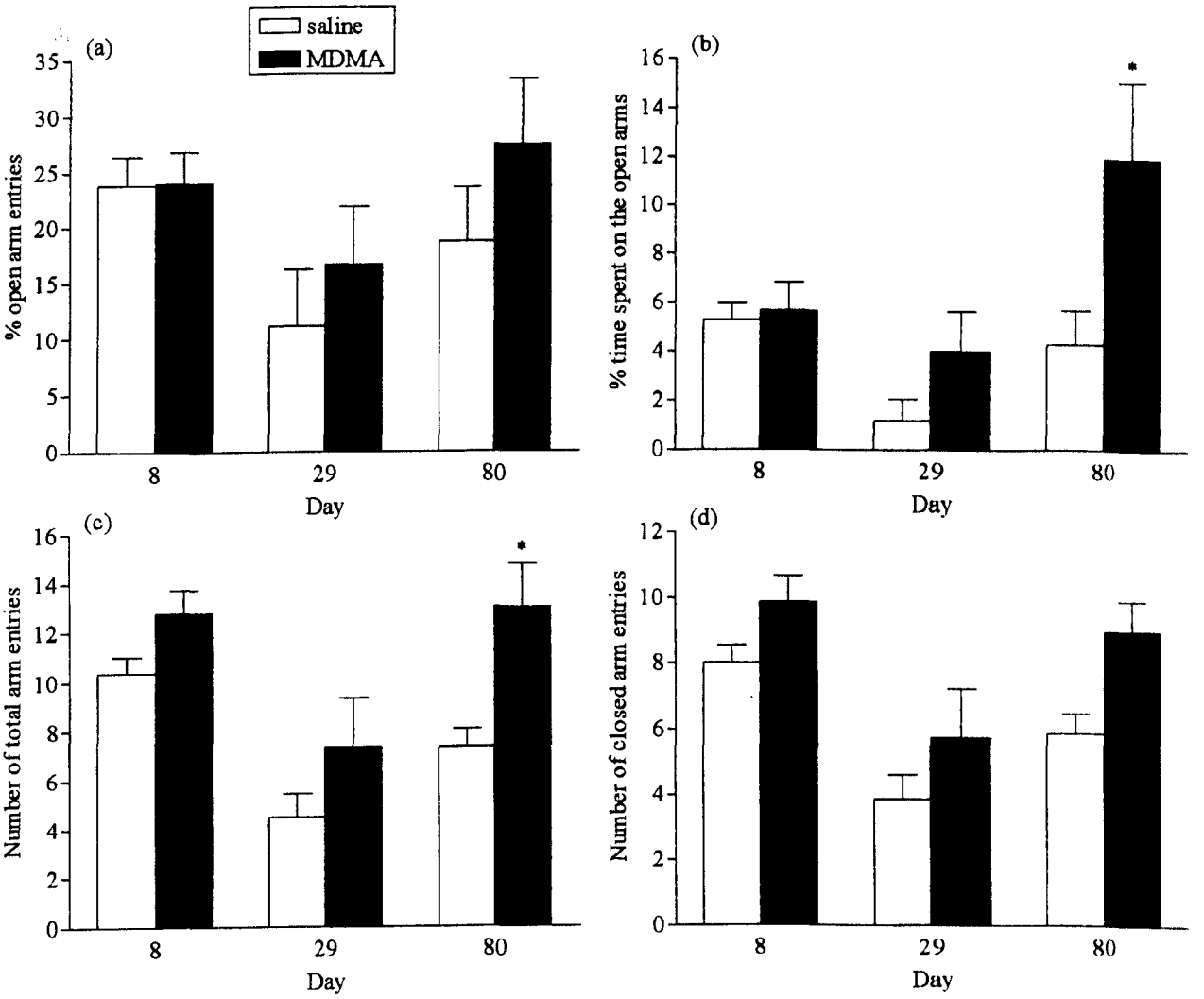

Figure 4.1 The behaviour of MDMA- and saline-pretreated DA rats on the elevated plus-maze.

Rats were administered a single dose of MDMA (12.5 mg/kg i.p.) or saline on Day 1. Results shown as mean ± s.e.m., n = 8 in each group. ***(a)*** *Open arm entries, calculated as a % of total arm entries.* There was no difference between treatment groups or between days. ***(b)*** *Time spent on the open arms, calculated as a % of time spent on all arms.* MDMA-pretreated rats were different from control rats ($F(1, 21) = 7.2$, $p < 0.05$), with post-hoc analysis demonstrating a statistical difference on Day 80 (*$p < 0.05$). Responses differed across the test days ($F(2, 21) = 5.3$, $p < 0.05$). ***(c)*** *Total arm entries.* MDMA-pretreated rats were different from control rats ($F(1, 21) = 9.2$, $p < 0.01$) and post-hoc analysis demonstrated a significant difference between treatment groups on Day 80 (*$p < 0.05$). Responses differed across the test days ($F(2, 21) = 15.2$, $p < 0.0001$). ***(d)*** *Closed arm entries.* MDMA-pretreated rats were different from control rats ($F(1, 21) = 8.4$, $p < 0.01$). Responses differed across the test days ($F(2, 21) = 13.2$, $p < 0.001$).

	Day 8		Day 29		Day 80		Two-way ANOVA		
	Saline	MDMA	Saline	MDMA	Saline	MDMA	TREATMENT	DAY	Interaction
Defecation	3.0 ± 1.0	3.5 ± 0.8	4.4 ± 0.8	5.6 ± 0.9	2.6 ± 1.2	2.4 ± 1.1	F (1, 21) = 0.4	F (2, 21) = 3.8*	F (2, 21) = 0.3
Grooming	4.4 ± 0.8	2.6 ± 0.2	3.3 ± 0.5	4.3 ± 0.4	4.6 ± 0.6	4.8 ± 0.5	F (1, 21) = 0.3	F (2, 21) = 2.4	F (2, 21) = 3.9$^{\Delta}$
Head dips	3.1 ± 0.7	4.5 ± 1.4	2.9 ± 1.1	4.5 ± 1.5	6.6 ± 0.9	10.0 ± 1.7	F (1, 21) = 3.3	F (2, 21) = 13.1***	F (2, 21) = 0.6
Rearing	26.3 ± 1.6	29.1 ± 3.5	22.9 ± 3.2	29.6 ± 5.7	27.9 ± 2.7	35.1 ± 1.5	F (1, 21) = 4.0	F (2, 21) = 1.4	F (2, 21) = 0.3
SAP	8.5 ± 0.9	10.0 ± 0.9	3.1 ± 0.9	3.4 ± 1.0	3.4 ± 0.6	5.0 ± 0.5	F (1, 21) = 2.7	F (2, 21) = 33.2***	F (2, 21) = 0.5
Sniffing (directed)	27.8 ± 2.1	33.4 ± 2.5	16.9 ± 2.2	18.6 ± 3.0	18.5 ± 1.4	20.6 ± 1.3	F (1, 21) = 2.9	F (2, 21) = 23.4***	F (2, 21) = 0.6
Sniffing (non-directed)	17.9 ± 1.3	18.4 ± 0. 8	19.5 ± 2.5	17.9 ± 2.8	19.3 ± 1.8	21.3 ± 1.6	F (1, 21) = 0.0	F (2, 21) = 0.6	F (2, 21) = 0.4

Table 4.1 Ethological variables measured in MDMA- and saline-pretreated DA rats on the elevated plus-maze.

Rats were administered a single dose of MDMA (12.5 mg/kg i.p.) or saline on Day 1. Results shown as mean ± s.e.m., n = 8 in each group. Responses were different on different days, where *$p < 0.05$ and ***$p < 0.001$; significant interaction between TREATMENT and DAY effects, where $^{\Delta}p < 0.05$.

4.3.3 Long-term effects of MDMA on the behaviour of DA rats in the open-field

Under white-light illumination (the first two days within each testing period), there were no differences between the behaviour of MDMA-pretreated and control animals with respect to anxiety-related behaviour (Zone 2 crossings, Figure 4.2a), locomotor behaviour (total zone crossings, Figure 4.2b), or ethological variables (Figure 4.3), thus no main effects of TREATMENT were observed. There were also no differences between days with respect to either Zone 2 or total numbers of crossings, thus no main effects of DAY were observed. Although the treatment groups did not differ with respect to ethological variables, there were observable differences between the test days in some of the behaviours monitored, resulting in main effects of DAY (Figure 4.3). For example, freezing behaviour increased in both treatment groups with each successive testing period, being approximately three times greater on Days 71 – 72 than on Days 9 – 10 ($p < 0.0001$), while both groups performed less rearing behaviour on Day 71 compared to all other white-light test days ($p < 0.01$).

Under red-light illumination (the third day of each testing period), differences between treatment groups became more apparent at the latest time-point (Day 73). MDMA-pretreated rats performed more Zone 2 crossings than control rats on both Day 32 and Day 73, although this effect was not statistically significant. Thus no main effect of TREATMENT was observed. MDMA-pretreated animals performed greater numbers of Zone 2 crossings on each successive test day compared to the previous test day, while there was very little difference in the response of control animals between test days (Figure 4.2c). However, this effect did not reach statistical significance, thus no main effect of DAY was seen. With respect to overall locomotor activity, indicated by total zone crossings (Figure 4.2d), MDMA-pretreated rats performed more crossings than control animals on each day, resulting in a main effect of TREATMENT, with post-hoc analysis highlighting the difference between groups on Day 73 ($p < 0.05$). MDMA-pretreated animals also performed more total crossings on each successive test day compared to the previous test day, but this effect was non-significant, thus neither an effect of DAY nor an interaction were observed.

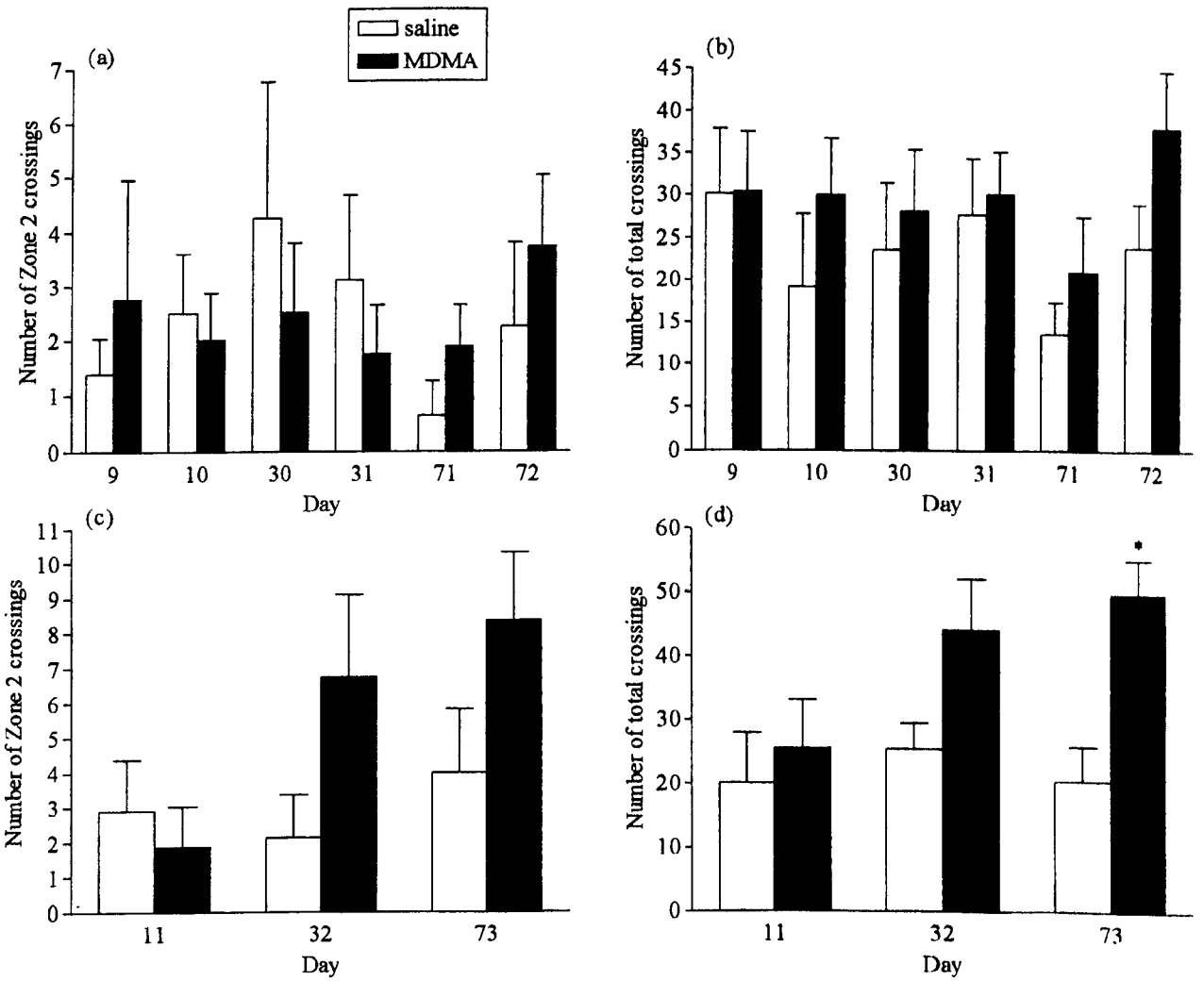

Figure 4.2 The behaviour of MDMA- and saline-pretreated DA rats in the open-field.

Rats were administered a single dose of MDMA (12.5 mg/kg i.p.) or saline on Day 1. Results shown as mean ± s.e.m., n = 8 in each group. ***(a)*** *Zone 2 crossings under white-light illumination.* There were no differences between treatment groups or between days. ***(b)*** *Total number of zone crossings under white-light illumination.* There were no differences between treatment groups or between days. ***(c)*** *Zone 2 crossings under red-light illumination.* There were no differences between treatment groups or between days. ***(d)*** *Total number of zone crossings under red-light illumination.* MDMA-pretreated rats were different from control rats ($F(1, 21) = 11.1$, $p < 0.01$) and post-hoc analysis demonstrated statistical significance on Day 73 ($^*p < 0.05$). There was no difference between days.

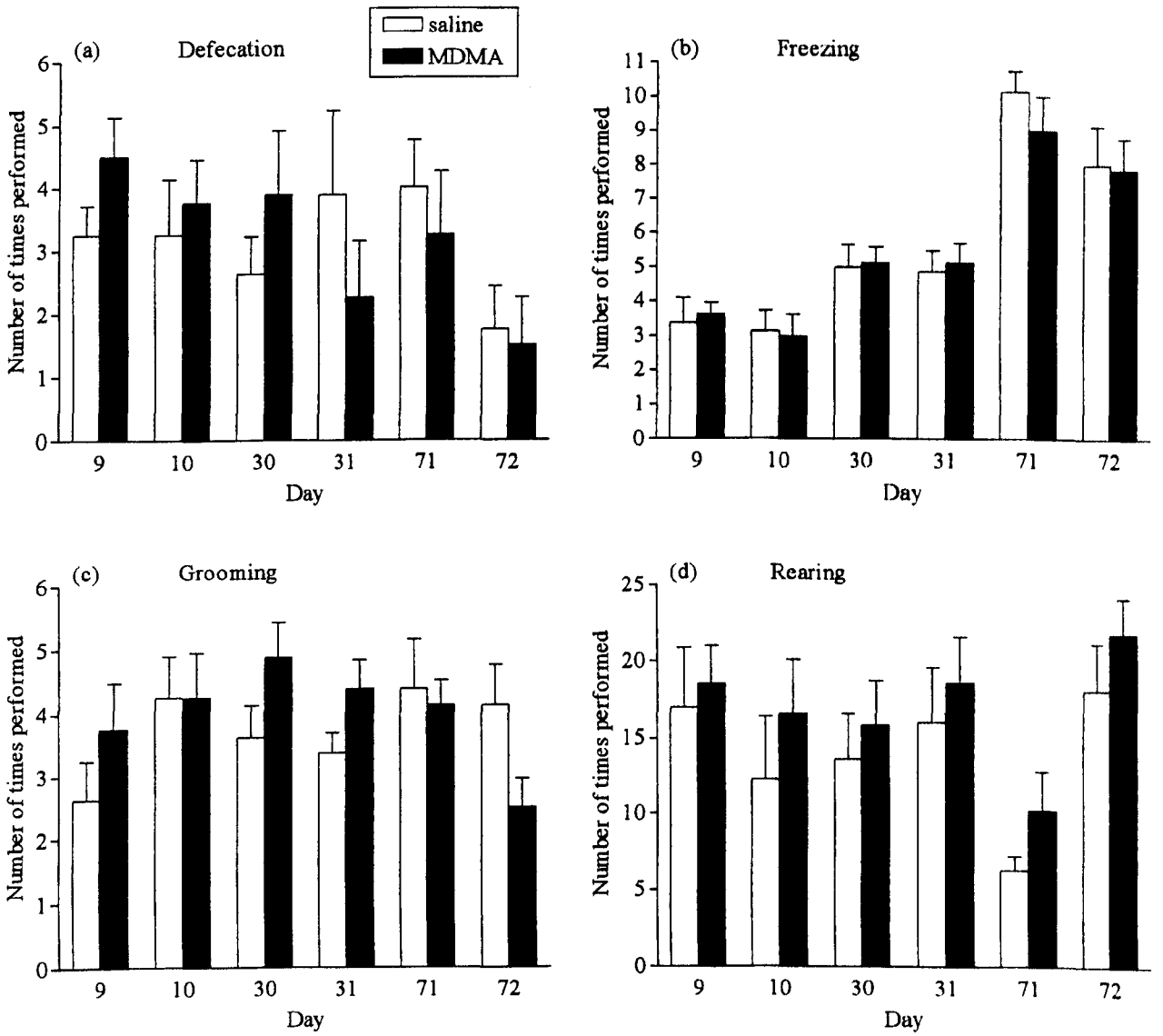

Figure 4.3 Ethological variables measured in MDMA- and saline-pretreated DA rats in the open-field, under white-light illumination.

Rats were administered a single dose of MDMA (12.5 mg/kg i.p.) or saline on Day 1. Results shown as mean ± s.e.m., n = 8 in each group. ***(a)*** *Defecation.* There were no differences between treatment groups or between days. ***(b)*** *Freezing.* There were no differences between treatment groups. Responses differed across the test days ($F(5, 42) = 17.9$, $p < 0.0001$). ***(c)*** *Grooming.* There were no differences between treatment groups or between days. ***(d)*** *Rearing.* There were no differences between treatment groups. Responses differed across the test days ($F(5, 42) = 4.2$, $p < 0.01$).

	Day 11		Day 32		Day 73		Two-way ANOVA		
	Saline	MDMA	Saline	MDMA	Saline	MDMA	TREATMENT	DAY	Interaction
Defecation	3.8 ± 0.7	5.3 ± 0.6	4.4 ± 1.0	5.1 ± 1. 7	4.0 ± 0.7	1.9 ± 1.0	F (1, 21) = 0.0	F (2, 21) = 1.7	F (2, 21) = 1.6
Freezing	2.8 ± 0.5	2.6 ± 0.5	7.0 ± 0.9	7.6 ± 1.0	6.8 ± 1.4	6.0 ± 0.9	F (1, 21) = 0.1	F (2, 21) = 12.4$^{\Delta\Delta\Delta}$	F (2, 21) = 0.3
Grooming	3.8 ± 1.1	4.3 ± 0.5	4.6 ± 0.7	3.6 ± 0.5	4.6 ± 0.7	4.1 ± 1.3	F (1, 21) = 0.2	F (2, 21) = 0.1	F (2, 21) = 0.5
Rearing	9.6 ± 3.3	13.4 ± 3.5	14.0 ± 2.0	18.6 ± 3.7	12.9 ± 2.4	21.0 ± 1.9	F (1, 21) = 5.7*	F (2, 21) = 2.0	F (2, 21) = 0.3

Table 4.2 Ethological variables measured in MDMA- and saline-pretreated DA rats in the open-field, under red-light illumination.

Rats were administered a single dose of MDMA (12.5 mg/kg i.p.) or saline on Day 1. Results shown as mean ± s.e.m., n = 8 in each group. MDMA-pretreated group different from saline-pretreated group, where *$p < 0.05$; responses were different on different days, where $^{\Delta\Delta\Delta}p < 0.001$.

Under red-light illumination, the majority of ethological variables did not differ between treatment groups on any of the test days (Table 4.2). The single exception was rearing behaviour, which was consistently greater in MDMA-pretreated rats compared to control animals and increased on each successive test day in the MDMA-pretreated group, resulting in a main effect of TREATMENT, but neither an effect of DAY nor an interaction. Freezing behaviour differed across the test days; both treatment groups performed more freezing behaviour on Days 32 and 73 compared to Day 11 ($p < 0.001$), which resulted in a main effect of DAY. Defecation behaviour remained consistent between the three red-light test days in saline-pretreated rats, while MDMA-pretreated rats defecated less on Day 73 compared to Days 11 and 32, although this effect was not significant. There were no differences in grooming behaviour within either treatment group between the three red-light test days.

4.4 DISCUSSION

This study demonstrated that a single neurotoxic dose of MDMA (which results in an approximately 20 - 40 % regional brain depletion of 5-HT and 5-HIAA - see section 5.3.3) has a long-term effect on anxiety-related and locomotor behaviour, as measured in the elevated plus-maze and open field.

On Day 8, one week after administration of MDMA or saline, there was no difference between treatment groups with regard to anxiety-related behaviours (% number of open arm entries and % time spent on the open arms). On Day 29, four weeks post-administration, there was no statistically significant difference between the treatment groups with regard to either of these measures. On Day 80, approximately 11 weeks after drug administration, MDMA-pretreated rats performed a greater, although non-statistically significant, % number of open arm entries compared to control animals. At this time-point, MDMA-pretreated rats spent a significantly greater % time on the open arms compared to control animals, the mean value being 177 % greater. Since the open arms of the elevated plus-maze are believed to evoke a "fear" response (see Montgomery, 1955; Handley & Mithani, 1984), the increase in open arm activity

expressed by MDMA-pretreated rats strongly indicates that these animals were less "anxious".

On all three test days, the MDMA-pretreated group displayed higher locomotor activity, as shown by greater numbers of total and closed arm entries. These differences became most apparent on Day 80, with MDMA-pretreated animals performing a statistically significant 75 % greater number of total arm entries and a 53 % greater number of closed arm entries, compared to control animals.

Both treatment groups demonstrated a decline in anxiety-related and locomotor behaviour on Day 29, compared to Days 8 and 80 which, with the exception of % number of open arm entries, resulted in statistically significant differences in responses between the different test days. Although the strain comparison study (see Chapter 3) did not demonstrate a significant habituation effect in naïve animals between their responses on Trial 2 and Trial 1, which were separated by 25 days, it seems likely that some habituation occurred in the current study. Here, both treatment groups differed in their behaviour in Trial 2 compared to Trial 1, which were separated by 21 days. There are two possible explanations for these observed differences: (1) the interval between trials is critical in determining whether or not habituation occurs, or (2) the single injection of saline seven days before Trial 1 has some bearing on the control group's behaviour. The first point seems an unlikely explanation for this result, as control animals tend not to demonstrate any differences in behaviour between trials (Pellow *et al.*, 1985; File *et al.*, 1990).

However, the second point requires further discussion. There were no clear differences between the responses of naïve animals (Figure 3.2) and the saline-pretreated rats in the current study (Figure 4.1), with the exception of the % number of open arm entries - saline-pretreated rats performed a greater % number of open arm entries on Day 8 (Trial 1) compared to naïve rats on Day 1 (23.8 ± 2.6 vs. 14.91 ± 4.5). However, there was no difference between naïve and saline-pretreated animals in Trial 1 with regard to the % time spent on the open arms or the numbers of total and closed arm entries. The effects of handling have been predominantly demonstrated in chronic studies, whereby rats are

handled and injected with water or saline daily for one or two weeks prior to being tested on the elevated plus-maze (Andrews & File, 1993; File *et al.*, 1992). However, a single handling/injecting experience is sufficient to alter the response of DA rats, as reported in Chapter 3, where an i.p. saline injection 30 min prior to exposure to the elevated plus-maze abolished the significantly greater anxiety-like behaviour of naïve DA rats, compared to naïve SD rats. In the current study, all animals were injected one week prior to Trial 1, where little difference was seen between naïve and saline-injected animals; differences only became apparent during Trial 2. Thus, in this case, perhaps there is some interaction between repeated testing and handling. Whatever the explanation for this phenomenon, control animals are obviously tested in parallel to drug-treated animals to enable a direct determination of the effects of the particular drug in question and any additional effects of repeated testing or handling should be consistent across the treatment groups.

With regard to the ethological variables measured on the elevated plus-maze, there were no significant differences between treatment groups. There were, however, some differences between the responses observed on different test days. For example, both treatment groups defecated most during Trial 2 (Day 29) and least on Day 80. Defecation is reported to signify increased "anxiety" or "emotionality" (Hall, 1934b), thus the reduction in this behaviour on Day 80 demonstrates a reduction in "anxiety" at this time-point. Although this is consistent with the reduction in other anxiety-related behaviours shown by MDMA-pretreated animals, such as the % time spent on the open arms, it does not explain the lack of difference between treatment groups. With regard to this measure, there was little difference between the responses of naïve DA rats (see Table 3.1) and either group in the current study (see Table 4.1). However, the numerical values involved were very low in all cases, which therefore makes significant effects of treatment difficult to detect. Both treatment groups performed increased numbers of head dips on Day 80, compared to Days 8 and 29. This behaviour is increased by anxiolytic drugs (Cruz *et al.*, 1994; Griebel *et al.*, 1997b), and therefore indicates reduced anxiety-like behaviour at the latest time-point. Both groups performed greater numbers of stretched attend postures and greater directed sniffing behaviour on Day 8 compared to the latter two trials, again indicating reduced anxiety-related behaviour at

the later time-points, these behaviours being indicative of risk assessment (Cruz *et al.*, 1994; Griebel *et al.*, 1997; Rodgers & Dalvi, 1997), which decreases in response to anxiolytic drugs (Griebel *et al.* 1997).

In the open-field under white-light illumination, there were no significant differences between the responses of MDMA- and saline-pretreated DA rats, with regard to anxiety-related or locomotor behaviour. This may have been because a brightly lit environment tends to induce approach-avoidance conflict behaviour, whereby animals avoid open, brightly illuminated spaces (Schmitt & Hiemke, 1998a), resulting in high levels of "anxiety" in both treatment groups. Red-light illumination, however, uncovered some differences between the behaviour of MDMA- and saline-pretreated rats in the open-field. There was little difference between the numbers of Zone 2 or total crossings performed by saline-pretreated rats on the different red-light test days, while MDMA-pretreated animals performed successively greater numbers of Zone 2 and total crossings on each test day. This resulted in behavioural differences between the two treatment groups, the total number of crossings reaching statistical significance on Day 73. It may be that red-light illumination provided a less anxiogenic environment, enabling some of the differences between the treatment groups to be unmasked, and is consistent with the results of Escorihuela *et al.* (1999) who demonstrated increased locomotor activity under "dark" (red-light illumination), compared to "light" (fluorescent lighting), conditions.

However, there was little difference between the two treatment groups with regard to the ethological variables measured, with the exception of rearing behaviour, which increased with successive test days in the MDMA-pretreated group and was consistently higher than that of the control group. Rearing is reflective of locomotor activity (Cruz *et al.*, 1994; File, 1992; Rodgers & Dalvi, 1997), and therefore the significantly higher levels seen in MDMA-pretreated animals (particularly at the later time-points) are consistent with the increased numbers of crossings performed by this group.

The majority of behavioural studies in MDMA-treated animals have been performed during the acute phase of action of the drug, which includes hyperactivity (Lin *et al.*,

1999; Marston *et al.*, 1999; McCreary *et al.*, 1999) and a stereotyped behavioural syndrome (Marston *et al.*, 1999). However, the reduction in anxiety-related behaviour seen in the current study is generally consistent with reported data where, for example: (1) acute MDMA administration (at higher doses, at least) has an anxiolytic effect on the behaviour of mice on the elevated plus-maze (Lin *et al.*, 1999); (2) chronic MDMA administration has an anxiolytic effect on the behaviour of rats in the open-field (McCreary *et al.*, 1999); and (3) acute and chronic amphetamine treatment have anxiolytic effects on the behaviour of rats on the elevated plus-maze (Olausson *et al.*, 2000). There are some exceptions, as McNamara *et al.* (1995) demonstrated no differences between the open-field behaviour of MDMA- and saline-treated rats three days after cessation of drug treatment.

The importance of the current findings should be put into context with regard to the effects of MDMA administration on brain 5-HT and the possible functional consequences of these effects in experimental animals and human recreational MDMA users. MDMA has been demonstrated to induce significant, long-lasting damage to central serotonergic systems in the brains of a number of different species (see Chapter 1). In rats, this damage is characterised by a selective degeneration of forebrain axon terminals (e.g. Commins *et al.*, 1987; O'Hearn *et al.*, 1988) and a decrease in 5-HT content (e.g. Colado *et al.*, 1995; Schmidt, 1987a) and [^{3}H]paroxetine binding (e.g. Colado *et al.*, 1995). Since disruptions in serotonergic activity induced by 5,7-DHT (e.g. Briley *et al.*, 1990; Hall *et al.*, 1999; Tye *et al.*, 1977) or PCPA (e.g. File & Hyde, 1977; Tye *et al.*, 1979) result in a reduction in anxiety-related behaviour, it might be expected that MDMA-induced serotonergic damage would have similar anxiolytic effects.

There is also evidence to suggest that human recreational users of MDMA exhibit serotonergic neurotoxicity, shown by a reduction in CSF 5-HIAA levels (McCann *et al.*, 1994) and a decrease in brain 5-HT transporter binding activity (McCann *et al.*, 1998). Psychological problems associated with 'Ecstasy' use have also been reported. For example, Parrott *et al.* (2000) noted an increase in anxiety, hostility and obsessional and impulsive behaviour in heavy 'Ecstasy' users (individuals who had taken 'Ecstasy' on

more than 20 occasions). However, as is often the case with human studies concerning illicit drug use, it is difficult to separate the effects of MDMA from either the potential effects of other drugs being used concurrently, such as alcohol, tobacco, cannabis and other amphetamines, or from pre-existing psychological conditions (Parrott *et al.*, 2000). McCann *et al.* (1999) subjected MDMA users (individuals who had taken 'Ecstasy' on at least 25 occasions) to an *m*-CPP challenge whereby the mixed 5-HT agonist and 5-HT-releasing compound, which has been demonstrated to increase anxiety in healthy individuals, was administered on Day 4 of a five day in-patient programme. MDMA users were significantly less sensitive to the anxiogenic effects of *m*-CPP than control subjects, shown by a greatly reduced occurrence of an *m*-CPP-induced panic attack. The authors suggested that this response was indicative of downregulation of postsynaptic 5-HT_{2C} receptors and that it may be a functional consequence of brain serotonergic neurotoxicity (McCann *et al.*, 1999). Therefore, although the anxiogenic or anxiolytic effects of MDMA use in humans have not yet been clarified, alterations in 5-HT activity are likely to have functional, possibly behavioural, consequences.

In conclusion, the effect of a single dose of MDMA (12.5 mg/kg i.p.), as used in the current study, has been demonstrated to induce a 20 - 40 % loss of 5-HT in all brain regions examined one week post-treatment (see section 5.3.3). Furthermore, this degree of serotonergic depletion has been shown to result in pronounced reductions in anxiety-related behaviour. The fact that such behavioural changes did not become apparent until over two months after a single dose of MDMA, while significant serotonergic depletions have been observed one week post-treatment, indicates that the behavioural changes observed are not solely due to serotonergic neurotoxicity, but that adaptive changes are occurring within the brain. In addition, these data highlight the potential damage incurred by human recreational users of MDMA - with recent evidence of serotonergic depletion (Kish *et al.*, 2000) and reduced densities of 5-HT transporters (McCann *et al.*, 1998; Ricaurte *et al.*, 2000) in the brains of chronic MDMA users, the fact that a relatively modest loss has long-term behavioural consequences in animals, suggests that high and/or frequent use may not be necessary to have long-lasting behavioural consequences in humans.

CHAPTER 5

TEMPERATURE I:

A STUDY OF THE LONG-TERM EFFECTS OF MDMA ADMINISTRATION ON THERMOREGULATION IN DARK AGOUTI RATS

5 TEMPERATURE I: A study of the long-term effects of MDMA administration on thermoregulation in Dark Agouti rats

5.1 INTRODUCTION

The acute effects of MDMA administration on temperature in rodents have been described in Chapter 1. The current chapter is concerned with the longer-term effects of MDMA on thermoregulation in DA rats. In particular, the effects of thermoregulatory "challenges" have been investigated in addition to a study of potential MDMA-induced changes in 5-HT receptor function.

5.1.1 Effects of MDMA administration on temperature: involvement of 5-HT

Long-term neurotoxic damage to brain serotonergic systems has been demonstrated to occur following administration of MDMA to rats (see Battaglia *et al.*, 1987; 1988; 1991; Colado *et al.*, 1995; 1997a; 1999a; Commins *et al.*, 1987; Molliver *et al.*, 1990; O'Hearn *et al.*, 1988; O'Shea *et al.*, 1998; Schmidt, 1987a; Schmidt *et al.*, 1987; Stone *et al.*, 1986), and following recreational use by humans (see Kish *et al.*, 2000; McCann *et al.*, 1994; 1998; Semple *et al.*, 1999). In addition, acute hyperthermia results from MDMA administration to rats (e.g. Broening *et al.*, 1995; Colado *et al.*, 1993; Dafters, 1994; 1995; Nash *et al.*, 1988; O'Shea *et al.*, 1998) and has been reported in human recreational users of MDMA, where temperatures of over 43 °C have been documented (e.g. Chadwick *et al.*, 1991; Randall, 1992; Screaton *et al.*, 1992). However, it should be noted that, in rats, ambient temperature plays an important role in MDMA-induced changes in body temperature - low ambient temperatures tend to induce hypothermic responses, while higher ambient temperatures induce hyperthermic responses (see Broening *et al.*, 1995; Dafters, 1994; 1995; Dafters & Lynch, 1998; Gordon *et al.*, 1991; Schmidt *et al.*, 1990a).

Since 5-HT is believed to be involved in temperature regulation (see Kruk & Pycock, 1991; Lucki, 1998; Milton, 1977; Myers, 1981; Rang *et al.*, 1995), MDMA-induced damage to serotonergic nerve endings might be expected to alter thermoregulatory

responses. Such changes could be potentially harmful to human recreational users of MDMA, particularly since MDMA is usually ingested in hot, crowded environments (see Green *et al.*, 1995; Henry *et al.*, 1992), which may further potentiate a hyperthermic response (see Henry *et al.*, 1992).

Salmi & Ahlenius (1998) investigated the involvement of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors in thermoregulation in male SD rats. Administration of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; 0.025 - 1.6 mg/kg s.c.) resulted in a dose-dependent hyperthermic response 20 min after injection, the maximal increase in rectal temperature being 1.1 °C following a dose of 0.4 mg/kg. Pretreatment with either 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; 0.05 mg/kg s.c.) or ritanserin (1 mg/kg s.c.) abolished the DOI-induced hyperthermia. Pretreatment with either of the 5-HT_{1A} receptor antagonists pindolol (0.5 - 1 mg/kg s.c.) or *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide 3HCl (WAY 100,635; 0.1 - 0.4 mg/kg s.c.), potentiated DOI-induced hyperthermia. This effect was abolished by pretreatment with the 5-$HT_{2A/2C}$ receptor antagonist ritanserin. The authors suggested that the potentiation of DOI-induced hyperthermia by WAY 100,635 could be due to the 5-HT_{1A} antagonist enhancing the response induced by DOI at 5-$HT_{2A/2C}$ postsynaptic receptors. Furthermore, the DOI/WAY 100,635-induced hyperthermic response was fully blocked by the selective 5-HT_{2A} antagonist amperozide, indicating an important role for 5-HT_{2A} receptors in rat thermoregulation (Salmi & Ahlenius, 1998).

Mazzola-Pomietto *et al.* (1995) demonstrated that DOI administration (1 mg/kg i.p.) to male Wistar rats resulted in a hyperthermic response (approximately 1.5 °C), which peaked within 1 h of drug administration. Pretreatment with the 5-HT antagonists metergoline, mesulergine, mianserin, ketanserin, LY53857, ritanserin or spiperone significantly attenuated the DOI-induced hyperthermic response. Mesulergine, mianserin, ketanserin, LY53857 and ritanserin are reported to have higher affinity for 5-HT_{2A} and 5-HT_{2C} receptors than other receptor subtypes. Spiperone is reported to have a higher affinity for 5-HT_{2A} receptors, compared to 5-HT_{2C} receptors, thus its blockade of

DOI-induced hyperthermia indicated the importance of 5-HT_{2A} receptors in mediation of this hyperthermic response (Mazzola-Pomietto *et al.*, 1995).

Mazzola-Pomietto *et al.* (1996) administered 1-(3-chlorophenyl)piperazine (*m*-CPP; 2.5 mg/kg i.p.) to male Wistar rats and demonstrated a hyperthermic response (approximately 1 °C), which peaked within 30 min of injection. Pretreatment with metergoline, mesulergine, mianserin, ketanserin, LY53857 or ritanserin significantly attenuated the *m*-CPP-induced hyperthermic response (Mazzola-Pomietto *et al.*, 1996). While rats did not become sensitised to the hyperthermic effects of DOI, within 17 consecutive days of drug administration (Mazzola-Pomietto *et al.*, 1995), tolerance to the hyperthermic effects of *m*-CPP developed within five days (Mazzola-Pomietto *et al.*, 1996). In addition, *m*-CPP-treated rats were not cross-tolerant to DOI; after five consecutive days of *m*-CPP treatment hyperthermia no longer occurred, yet a single challenge dose of DOI induced a hyperthermic response. These results indicated that *m*-CPP and DOI-induced hyperthermia are mediated by different 5-HT receptors and, since both compounds have antagonistic actions at 5-HT_{2A} and 5-HT_{2C} receptors, while DOI-induced hyperthermia is believed to be mediated by 5-HT_{2A} receptors, the authors suggested that *m*-CPP-induced hyperthermia is mediated by 5-HT_{2C} receptors (Mazzola-Pomietto *et al.*, 1996).

5.1.2 Aims of the investigation

Several reported studies have suggested that administration of neurotoxic doses of MDMA results in long-term functional changes in thermoregulation and 5-HT receptor activity (see below). Thus, the current study was undertaken as a further investigation into both of these areas. In each case, MDMA was administered to male DA rats at a neurotoxic dose of 12.5 mg/kg i.p.

5.1.2.1 "Thermoregulatory challenges"

Dafters & Lynch (1998) investigated the effects of a "thermoregulatory challenge" on the body temperature of rats administered MDMA (10 mg/kg s.c., daily for four consecutive days), under ambient temperature (T_a) conditions of 22 or 28 °C. Animals were exposed to a high T_a environment (30 °C) for 1 h ("thermoregulatory challenge")

on three separate occasions: (1) one week prior to MDMA treatment, (2) four weeks post-treatment, and (3) 14 weeks post-treatment. On the pre-drug test day, body temperature increased by approximately 2 °C, reaching a maximum within 60 min after commencement of the "thermoregulatory challenge", and returning to pre-challenge values 50 min later (110 min after the start of the challenge). Four weeks after drug administration, body temperature increased by approximately 1.5 °C, being maximal within 40 min after the start of the challenge, and returning to pre-challenge values a further 120 min later. Fourteen weeks post-treatment, body temperature increased by approximately 1.6 °C, within 30 min of the beginning of the challenge, and returned to pre-challenge values 180 min later. Thus, the hyperthermic response induced by exposure to a high T_a was prolonged after administration of MDMA; 14 weeks post-treatment, this response lasted for over 1.5 h after the end of the 60 min challenge. The authors concluded that such a response to an environmental challenge indicated relatively permanent changes in the neural mechanisms involved in thermoregulation, and that these changes might be indicative of serotonergic neurotoxicity (Dafters & Lynch, 1998).

There were some difficulties in interpreting the data of Dafters & Lynch (1998), in particular: (1) a parallel vehicle-treated control group was not used; the same animals were used in the "thermoregulatory challenge" prior to and following MDMA administration. This meant that, instead of being able to directly compare the temperature responses of MDMA-treated with control animals during each test, the responses were compared to those recorded at the same time during the previous test, which resulted in a somewhat confusing presentation of data; (2) although MDMA was administered at 14:00 h on each treatment day (a time which was chosen to minimise any effects of the diurnal temperature cycle - see Dafters, 1994), the thermoregulatory challenges were performed at different times of the day on each occasion; the pre-drug challenge commenced at 13:00 h, the four week post-drug challenge commenced at 10:00 h, and the 14 week post-drug challenge commenced at 09:30 h. Thus any potential effects of diurnal rhythms on thermoregulatory mechanisms were not accounted for in this part of the study; (3) the authors suggested that the observed impairments in thermoregulation were a manifestation of serotonergic neurotoxicity, yet

did not assess brain concentration of 5-HT in the animals. Therefore, any relationship between the extent of serotonergic damage and the long-term effects of MDMA administration on thermoregulation could not be determined.

The current study repeated the work of Dafters & Lynch (1998), to some extent, and comprised: (1) administration of a single dose of MDMA, (2) two "thermoregulatory challenges", involving exposure of the animals to a high (30 ± 0.5 °C) or a low (10 ± 0.5 °C) T_a environment for 60 min, five to six weeks post-treatment, and (3) h.p.l.c. analysis of regional brain concentrations of 5-HT and 5-HIAA. In addition, conventional rectal temperature measurement was performed, rather than the biotelemetric temperature measuring devices used by Dafters & Lynch (1998).

5.1.2.2 Effects of MDMA pretreatment on MDMA-induced hyperthermia

Shankaran & Gudelsky (1999) treated male SD rats with a neurotoxic regimen of MDMA (10 mg/kg i.p., four times at 2 h intervals), and injected a further single dose of MDMA (7.5, 10 or 20 mg/kg) one week later. A dose-dependent hyperthermic response was observed in both MDMA- and vehicle-pretreated animals; the increase in body temperature following a single dose of MDMA to vehicle-pretreated rats ranged from 1.2 °C (7.5 mg/kg) to 2.2 °C (20 mg/kg). Following injection of all three doses of MDMA, the hyperthermic response seen in MDMA-pretreated animals was less than that seen in vehicle-pretreated rats. This effect was statistically significant following MDMA doses of 7.5 and 10 mg/kg, where the MDMA-induced hyperthermic responses in MDMA-pretreated rats were approximately 58 % and 35 %, respectively, less than the responses seen in vehicle-pretreated rats. The authors suggested that the attenuated hyperthermic responses to low doses of MDMA in rats previously treated with a neurotoxic regimen of MDMA, might indicate attenuation of stimulated 5-HT release. Administration of a higher dose of MDMA (20 mg/kg), however, might evoke the release of 5-HT to stimulate sufficient 5-HT_2 receptors, and thus produce a maximal hyperthermia. The authors also suggested that the diminished MDMA-induced hyperthermic response to a subsequent dose of MDMA, might reflect an up-regulation of 5-HT_{1A} receptors (stimulation of which can induce hypothermia), thus antagonising a 5-HT_2 receptor-mediated hyperthermic response. Furthermore, it was suggested that

MDMA-induced depletion of 5-HT might result in functional deficits in serotonergic neurotransmission (Shankaran & Gudelsky, 1999).

The current study involved administration of a single neurotoxic dose of MDMA or saline, followed by second and third doses of MDMA (12.5 mg/kg i.p.) administered to all rats, approximately three weeks and five weeks after the first dose, respectively.

5.1.2.3 5-HT_{1A} receptor function

Aguirre *et al.* (1998) demonstrated a significant increase in postsynaptic 5-HT_{1A} receptor density in the frontal cortex and hypothalamus, and a significant decrease in regional brain 5-HT content, of male Wistar rats one week after administration of MDMA (30 mg/kg i.p.). As shown by Goodwin *et al.* (1987), administration of 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; 1 mg/kg s.c.) resulted in a hypothermic response of approximately 1.75 °C in control animals. This effect was potentiated in animals which had received MDMA (30 mg/kg i.p., twice each day for four consecutive days) seven days earlier; MDMA-treated animals demonstrated a drop in temperature of approximately 2.75 °C. Pretreatment with fluoxetine, ketanserin or haloperidol prevented MDMA-induced loss of 5-HT content, and fluoxetine and ketanserin prevented MDMA-induced increases in 5-HT_{1A} receptor density. None of the three pretreatment compounds modified 8-OH-DPAT-induced hypothermia. These results indicated that MDMA administration induced adaptive changes in postsynaptic 5-HT_{1A} receptors (Aguirre *et al.*, 1998).

In contrast, McNamara *et al.* (1995) did not demonstrate any effect of MDMA pretreatment on 8-OH-DPAT-induced hypothermia. MDMA was administered to male SD rats (5, 10 or 20 mg/kg i.p., twice daily for four consecutive days) and, four days after the last MDMA injection, there was no significant difference in the rectal temperature of the treatment groups. On Day 8, administration of 8-OH-DPAT (0.15 mg/kg s.c.) resulted in a hypothermic response, which comprised a drop in rectal temperature of approximately 3 °C, 30 min post-injection. None of the doses of MDMA had any significant effect on the magnitude of the 8-OH-DPAT-induced hypothermic response (McNamara *et al.*, 1995).

As conflicting results have previously been reported, the current study was performed and involved: (1) administration of a single dose of MDMA or saline, and (2) three to four weeks later, 8-OH-DPAT was administered (0.057, 0.09 and 0.11 mg/kg s.c.) to all animals and their temperature response monitored.

5.1.2.4 5-HT_{2A} and 5-HT_{2C} receptor function

Administration of DOI (Mazzola-Pomietto *et al.*, 1995; Salmi & Ahlenius, 1998) and *m*-CPP (Mazzola-Pomietto *et al.* 1996) have been demonstrated to induce acute hyperthermic responses, which were believed to be mediated by the 5-HT_{2A} receptor and the 5-HT_{2C} receptor, respectively (Mazzola-Pomietto *et al.*, 1995; 1996). Thus the current study comprised administration of both of these compounds to MDMA-naïve DA rats and monitoring of rectal temperature. It was planned that a subsequent study would involve investigation of DOI- and *m*-CPP-induced changes in rectal temperature, in MDMA-pretreated animals.

5.2 METHODS

5.2.1 Animals and drug administration

Male DA rats were housed as detailed in section 2.1(1). MDMA was administered at a dose of 12.5 mg/kg i.p. and 8-OH-DPAT was administered at doses of 0.057, 0.09 and 0.11 mg/kg s.c.

5.2.2 Temperature and weight measurement

Rectal temperature was measured, as detailed in section 2.7, at regular intervals during all experiments. In one group of rats, rectal temperature was also monitored every one or two days (at 10:00 – 11:00 h) preceding and succeeding MDMA administration, in order to determine whether MDMA had any long-lasting effect on rectal temperature under 'normal' (20 ± 2 °C) ambient temperature conditions. Weight was also monitored in the latter group of rats every one or two days, to determine whether MDMA had any effect on normal weight gain.

5.2.3 Acute temperature response to MDMA administration

MDMA (12.5 mg/kg i.p.) or saline (n = 12 in each group) was administered to a group of rats at 10:00 - 11:00 h. Rectal temperature was measured prior to injection and at regular intervals thereafter, for a total of 3.5 h.

5.2.4 Measurement of 5-HT and 5-HIAA in regional brain tissue

A separate group of rats, administered MDMA (12.5 mg/kg i.p.) or saline (n = 7 - 10 in each group) one week previously, were sacrificed and their brains dissected to provide cortical, hippocampal, striatal and hypothalamic tissue samples (see section 2.8 for details). 5-HT and 5-HIAA levels in these brain regions were subsequently measured using h.p.l.c. with electrochemical detection (see section 2.9 for details).

5.2.5 Thermoregulatory challenges

5.2.5.1 High ambient temperature

Five to six weeks following administration of MDMA (12.5 mg/kg i.p.) or saline, all animals (n = 12 in each treatment group) were subjected to a thermoregulatory challenge. Rectal temperature was recorded in a 'normal' ambient temperature environment (20 ± 2 °C), 10 min and immediately prior to commencement of the challenge. The rats were then placed in a high ambient temperature environment (30 ± 0.5 °C) for 60 min. Rectal temperature was monitored at regular intervals during the challenge and for a further 2.5 h when the animals had been returned to the 'normal' environment.

5.2.5.2 Low ambient temperature

MDMA-pretreated (12.5 mg/kg i.p.) and control animals were also subjected to a low ambient temperature challenge (n = 6 in each group). Rectal temperature was again measured prior to, during, and after the challenge, which consisted of a 60 min exposure to a low ambient temperature environment (10 ± 0.5 °C).

5.2.6 Effect of pretreatment with MDMA on the hyperthermic response of rats to subsequent doses of MDMA

A separate group of rats were administered MDMA (12.5 mg/kg i.p) or saline (n = 5 - 6 in each group) and their acute temperature response monitored. Approximately three weeks later, both groups were administered MDMA (12.5 mg/kg i.p.) and their acute temperature response again measured. Approximately two weeks after the second dose, both groups were administered MDMA (12.5 mg/kg i.p.) and their temperature monitored as before.

5.2.7 Long-term effects of acute MDMA administration: 5-HT_{1A} receptor function

Three to four weeks following MDMA administration (12.5 mg/kg i.p.), MDMA-pretreated and control rats (n = 6 in each group) were administered the 5-HT_{1A} receptor agonist 8-OH-DPAT (0.11 mg/kg s.c.). Rectal temperature was recorded at regular intervals throughout the experiment. Two further doses of 8-OH-DPAT (0.057 and 0.090 mg/kg s.c.) were administered to MDMA- and saline-pretreated rats (n = 6 in each group) and temperature was monitored.

5.2.8 Acute temperature response to administration of 5-HT_{2A} and 5-HT_{2C} receptor agonists

The 5-HT_{2A} agonist (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI; 1 - 2 mg/kg i.p.) and the 5-HT_{2C} agonist 1-(3-chlorophenyl)piperazine (*m*-CPP; 1.5 - 10 mg/kg i.p.) were administered to a group of rats (n = 4) and rectal temperature was monitored prior to and following injection, for a total of 90 min.

5.2.9 Statistics

Two-way ANOVA with repeated measures was performed on all temperature data, with TREATMENT (MDMA or saline) as the between subjects factor and TIME (or DAY, for analysis of day-to-day temperature and weight data, or DOSE for analysis of 8-OH-DPAT data) as the repeated measure. Indole concentrations were analysed by student's unpaired *t*-tests.

5.3 RESULTS

5.3.1 Acute temperature response to MDMA administration

Following administration of a single dose of MDMA (12.5 mg/kg i.p.) rectal temperature was increased by approximately 1.5 – 2 °C compared to control animals, peaking at 60 min (Figure 5.1) and being sustained for over 3.5 h. This resulted in main effects of both TREATMENT and TIME and a significant interaction between these effects ($F(7, 88) = 4.4$, $p < 0.001$).

5.3.2 Effects of MDMA administration on rectal temperature and body weight

MDMA administration had no effect on day-to-day rectal temperature, as measured at the same time (10:00 – 11:00 h) and same ambient temperature (20 ± 2 °C) on each occasion (Figure 5.2a), therefore there was no main effect of TREATMENT. However, there were differences in temperature between days prior to treatment, within both treatment groups, thus a main effect of DAY was observed but no interaction.

The two treatment groups did differ in their day-to-day weight gain (Figure 5.2b), the saline treatment group being of greater weight both prior to and following the injection day. Both groups gained weight over the whole measurement period resulting in a main effect of DAY. Although the MDMA treatment group demonstrated a small drop in weight on the day after injection and both groups demonstrated a drop in weight on Day 26 (Figures 5.2b and 5.2c), there was no difference between the two treatment groups where weight was calculated as a % of pretreatment values (Figure 5.2c).

5.3.3 Effects of MDMA administration on 5-HT and 5-HIAA levels in regional brain tissue

MDMA administration resulted in a loss of both 5-HT and 5-HIAA in all brain regions examined one week post-treatment (Table 5.1). In particular, there were statistically significant reductions in 5-HT concentration of 41 % in the hypothalamus and 21 % in the striatum ($p < 0.05$).

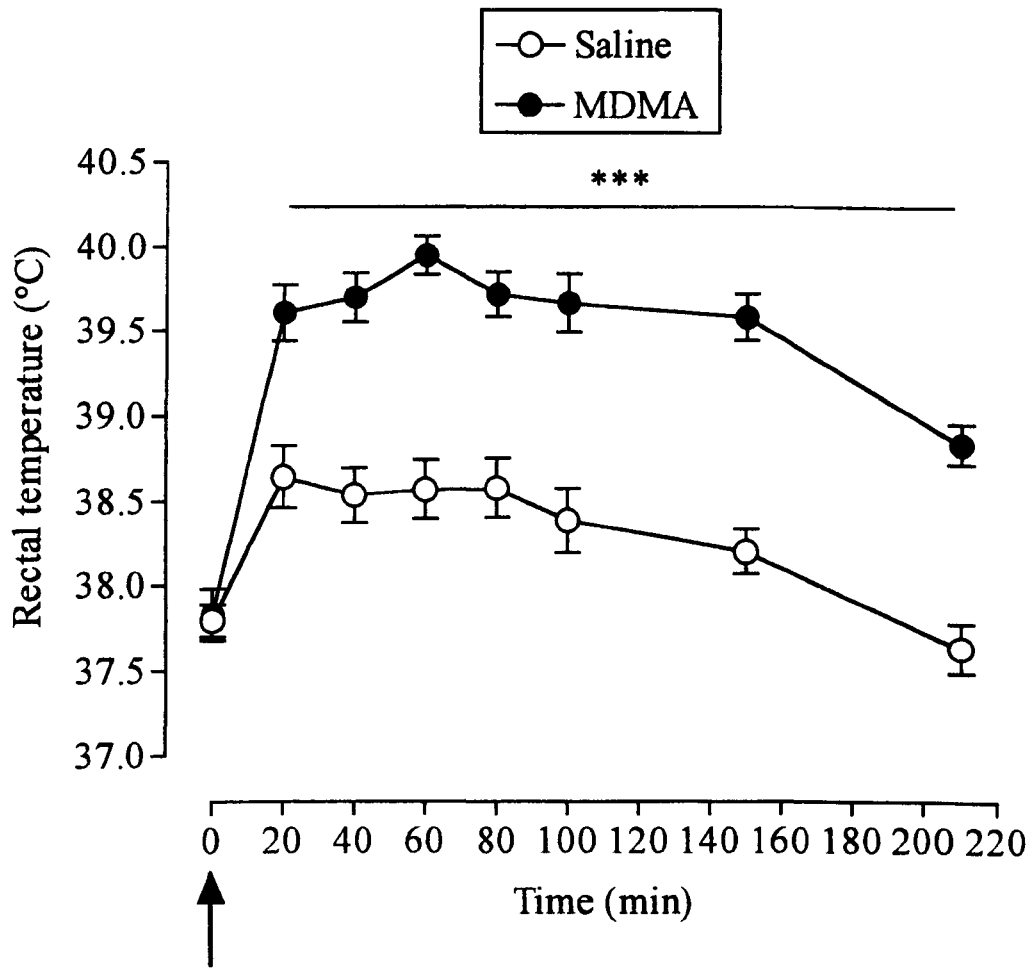

Figure 5.1 Acute effect of MDMA administration on rectal temperature.

Rats were administered MDMA (12.5 mg/kg i.p.) or saline at t = 0 (denoted by arrow). Results shown as mean ± s.e.m., n = 12 in each group. MDMA-treated rats were different from control rats ($F(1, 88) = 187$, $p < 0.0001$) with post-hoc analysis showing statistical significance during the period t_{20-210} (***$p < 0.001$). Rectal temperature changed over time ($F(7, 88) = 24.7$, $p < 0.0001$).

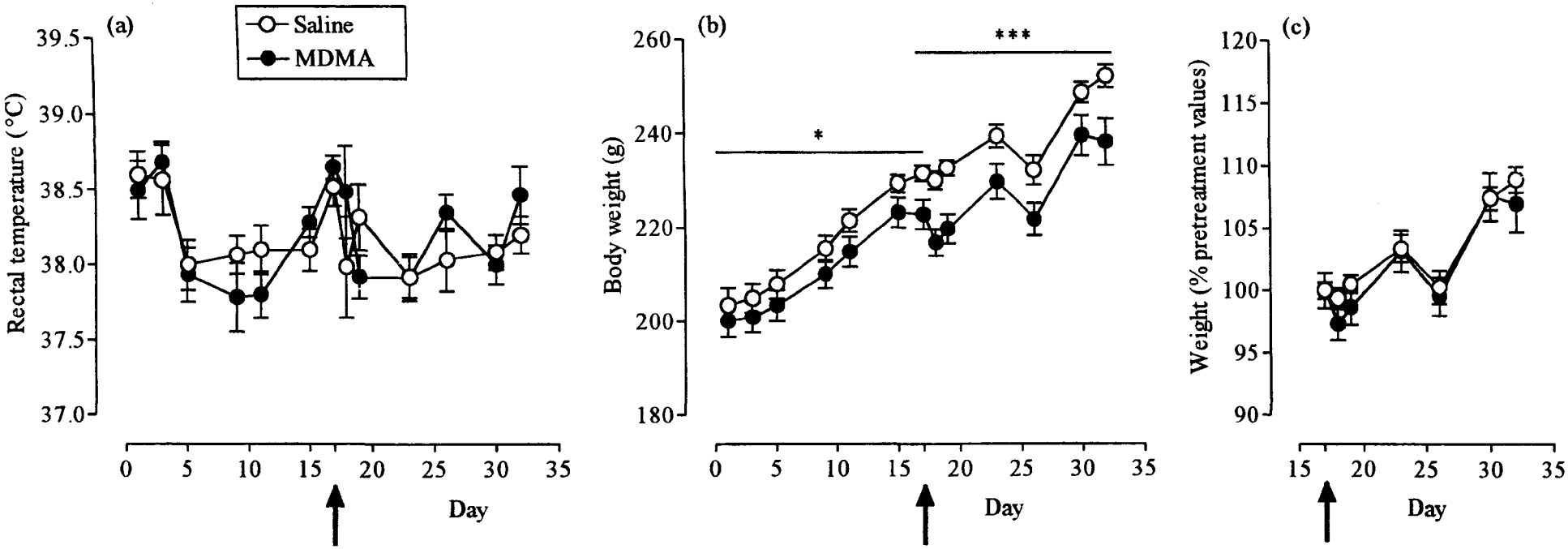

Figure 5.2 Rectal temperature and body weight of a group of rats, measured every 1 - 2 days preceding and succeeding MDMA administration.

Results shown as mean ± s.e.m., n = 6 in each group. MDMA (12.5 mg/kg i.p.) or saline was administered on Day 17 (denoted by arrows). ***(a) Rectal temperature.*** There was no difference between treatment groups, prior to or following MDMA/saline administration. There was a difference in temperature between days prior to injection (Days 1 - 17: $F(4, 25) = 5.6$, $p < 0.01$) ***(b) Body weight.*** MDMA-treatment group was different from the control group both prior to (Days 1 – 17: $F(1, 35) = 6.9$, $^{*}p < 0.05$) and following (Days 18 – 32: $F(1, 30) = 23.2$, $^{***}p < 0.0001$) injection. Body weight increased over the measurement period (Days 1 - 17: $F(6, 35) = 130.4$, $p < 0.0001$; Days 18 - 32: $F(5, 30) = 73.5$, $p < 0.0001$). ***(c) Body weight of animals on days following treatment, calculated as a % of weight immediately prior to injection.*** There was no difference between treatment groups.

	Indole concentration (ng/g tissue)					
	Indole	Saline	MDMA	% loss	t	p
Hypothalamus	5-HT	310 ± 24	184 ± 33	41	2.9	0.01*
	5-HIAA	348 ± 42	227 ± 44	35	1.9	0.08
Hippocampus	5-HT	197 ± 29	133 ± 18	32	1.9	0.07
	5-HIAA	265 ± 27	219 ± 24	18	1.3	0.21
Cortex	5-HT	242 ± 27	191 ± 32	21	1.2	0.26
	5-HIAA	215 ± 12	170 ± 18	21	2.0	0.06
Striatum	5-HT	316 ± 20	250 ± 22	21	2.2	0.04*
	5-HIAA	407 ± 20	343 ± 24	16	2.0	0.06

Table 5.1 Regional brain 5-HT and 5-HIAA levels measured 1 week after administration of MDMA.

Rats were administered MDMA (12.5 mg/kg i.p.) or saline and were sacrificed 1 week later for measurement of 5-HT and 5-HIAA. Results shown as mean ± s.e.m., n = 7 -10 in each group. MDMA-treated rats demonstrated a significant loss of 5-HT where *$p < 0.05$.

5.3.4 Thermoregulatory challenges

5.3.4.1 High ambient temperature

Five to six weeks after MDMA administration, there was no difference between the rectal temperature of the MDMA-pretreated and control animals. When both groups were subjected to a high ambient temperature environment (30 ± 0.5 °C) for 60 min, the MDMA-pretreated group demonstrated a faster rise in rectal temperature than the control group (Figure 5.3), resulting in a main effect of TREATMENT.

The rectal temperature of both MDMA- and saline-pretreated animals increased during the high ambient temperature challenge. A maximal change in temperature, compared to that at t = 0, was reached at t = 40 in the MDMA-pretreated animals ($\Delta T = 0.69$ °C) and at t = 60 in the control group ($\Delta T = 0.77$ °C). Thus there was a main effect of TIME but no interaction between TREATMENT and TIME.

When the rats were returned to a 'normal' (20 ± 2 °C) environment, the MDMA-pretreated animals demonstrated a prolonged hyperthermic response compared to the control group, resulting in a main effect of TREATMENT. The rectal temperature of both groups decreased over time, thus a main effect of TIME was observed in addition to an interaction between the effects of TREATMENT and TIME ($F(6, 77) = 3.3$, $p < 0.01$).

5.3.4.2 Low ambient temperature

Prior to commencement of the low ambient temperature challenge (10 ± 0.5 °C for 60 min) both MDMA- and saline-pretreated groups demonstrated an initial temperature rise of approximately 1 °C (Figure 5.4).

Subsequently, both during and after the challenge, the rectal temperature of both groups steadily declined towards pre-challenge values, resulting in a main effect of TIME. The temperature of MDMA-pretreated animals remained higher than that of the control animals throughout the period t_{0-180}, resulting in main effects of TREATMENT (Figure 5.4). There was no interaction between the effects of TREATMENT and TIME.

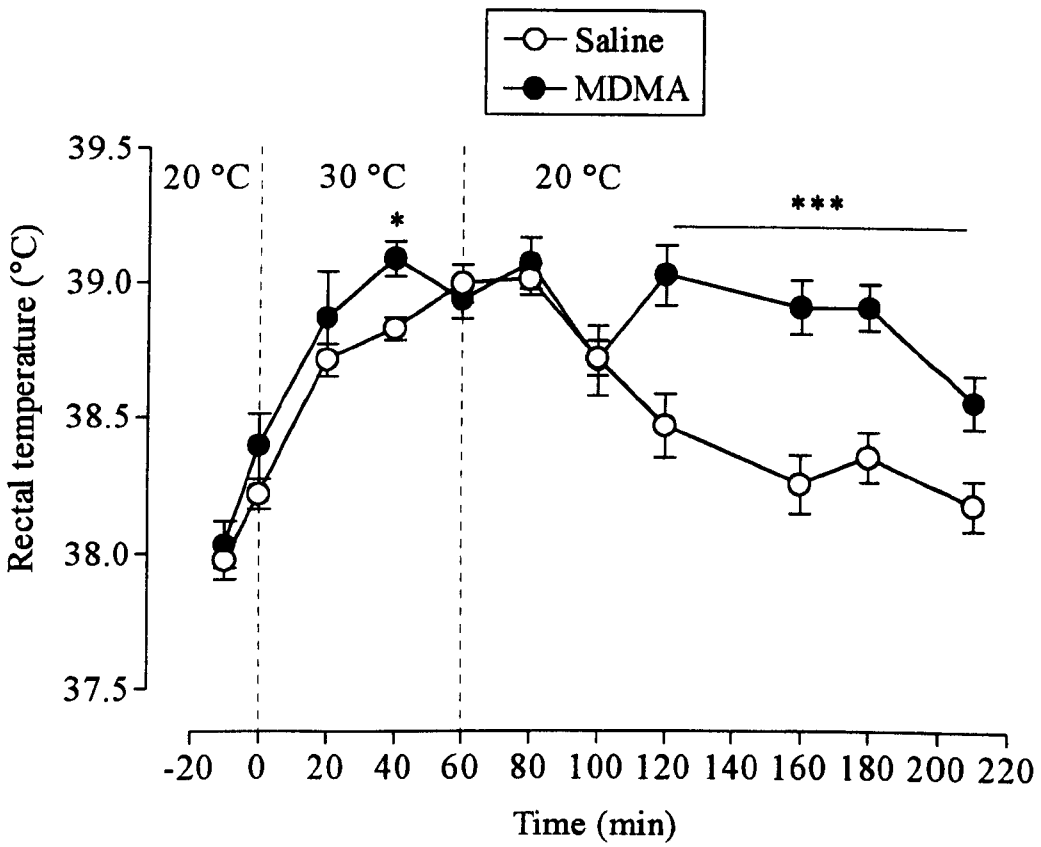

Figure 5.3 Rectal temperature of a group of rats subjected to a high ambient temperature thermoregulatory challenge, 5 - 6 weeks following administration of MDMA.

Rats were subjected to a high ambient temperature environment (30 ± 0.5 °C) for 60 min, 5 – 6 weeks after administration of a single dose of MDMA (12.5 mg/kg i.p.) or saline. Results shown as mean ± s.e.m., n = 12 in each group. MDMA-pretreated rats were different from control rats both during the challenge ($t_{0\text{-}60}$: $F(1, 44) = 8.8$, $p < 0.01$) and when the rats had been returned to a 'normal' (20 ± 2 °C) environment ($t_{60\text{-}210}$: $F(1, 77) = 72.2$, $p < 0.0001$). Post-hoc analysis demonstrated a significant difference between treatment groups, where $^{*}p < 0.05$ and $^{***}p < 0.001$. Rectal temperature changed over time ($t_{0\text{-}60}$: $F(3, 44) = 15.9$, $p < 0.0001$; $t_{60\text{-}210}$: $F(6, 77) = 7.8$, $p < 0.0001$).

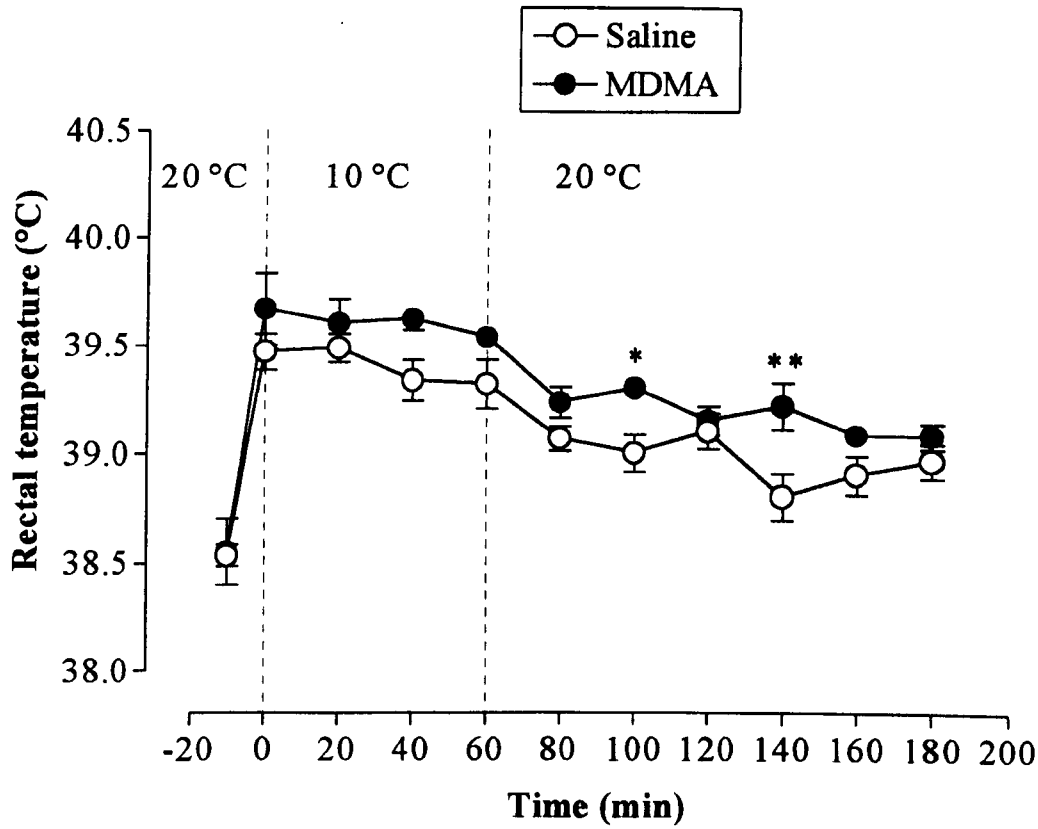

Figure 5.4 Rectal temperature of a group of rats subjected to a low ambient temperature thermoregulatory challenge, 5 - 6 weeks following administration of MDMA.

Rats were subjected to a low ambient temperature environment (10 ± 0.5 °C) for 60 min, 5 – 6 weeks after administration of a single dose of MDMA (12.5 mg/kg i.p.) or saline. Results shown as mean ± s.e.m., n = 6 in each group. MDMA-pretreated rats were different from control rats both during the challenge ($t_{0\text{-}60}$: $F(1, 20) = 10.3$, $p < 0.01$) and when the rats had been returned to a 'normal' (20 ± 2 °C) environment ($t_{60\text{-}180}$: $F(1, 35) = 29.2$, $p < 0.0001$). Post-hoc analysis demonstrated a significant difference between treatment groups, where $^{*}p < 0.05$ and $^{**}p < 0.01$. Rectal temperature changed over time ($t_{60\text{-}180}$: $F(6, 35) = 6.5$, $p < 0.001$).

5.3.5 Effect of pretreatment with MDMA on the hyperthermic response of rats to subsequent doses of MDMA

A group of rats that had been pretreated 23 days earlier with either MDMA (12.5 mg/kg i.p.) or saline were both injected with MDMA (12.5 mg/kg i.p.). The resulting hyperthermic response was similar in both groups (Figure 5.5). Twelve days later, all rats were again challenged with MDMA (12.5 mg/kg i.p.), the resulting hyperthermic response being similar to that seen after the previous doses (Figure 5.5). There was no difference in the hyperthermic responses seen between the three different MDMA-treated groups.

5.3.6 Long-term effects of acute MDMA administration: 5-HT_{1A} receptor function

Administration of 8-OH-DPAT (0.11 mg/kg s.c.) induced an acute hypothermic response in both MDMA- and saline-pretreated animals, reaching a nadir at 25 min post-injection (Figure 5.6a) and resulting in a main effect of TIME. The rectal temperature of MDMA-pretreated animals remained marginally higher than that of the saline-pretreated animals throughout the experiment, resulting in a main effect of TREATMENT. There was no interaction between the effects of TREATMENT and TIME.

A similar response was apparent at all three doses of 8-OH-DPAT investigated (Figure 5.6b); there was no difference in the change in temperature at the nadir (ΔT at 25 min) between MDMA- and saline-pretreated rats, thus no main effect of TREATMENT was observed at this time-point.

5.3.7 Acute temperature response to administration of 5-HT_{2A} and 5-HT_{2C} receptor agonists

Neither the 5-HT_{2A} agonist DOI, nor the 5-HT_{2C} agonist *m*-CPP had any significant hyperthermic effect on rectal temperature (Table 5.2).

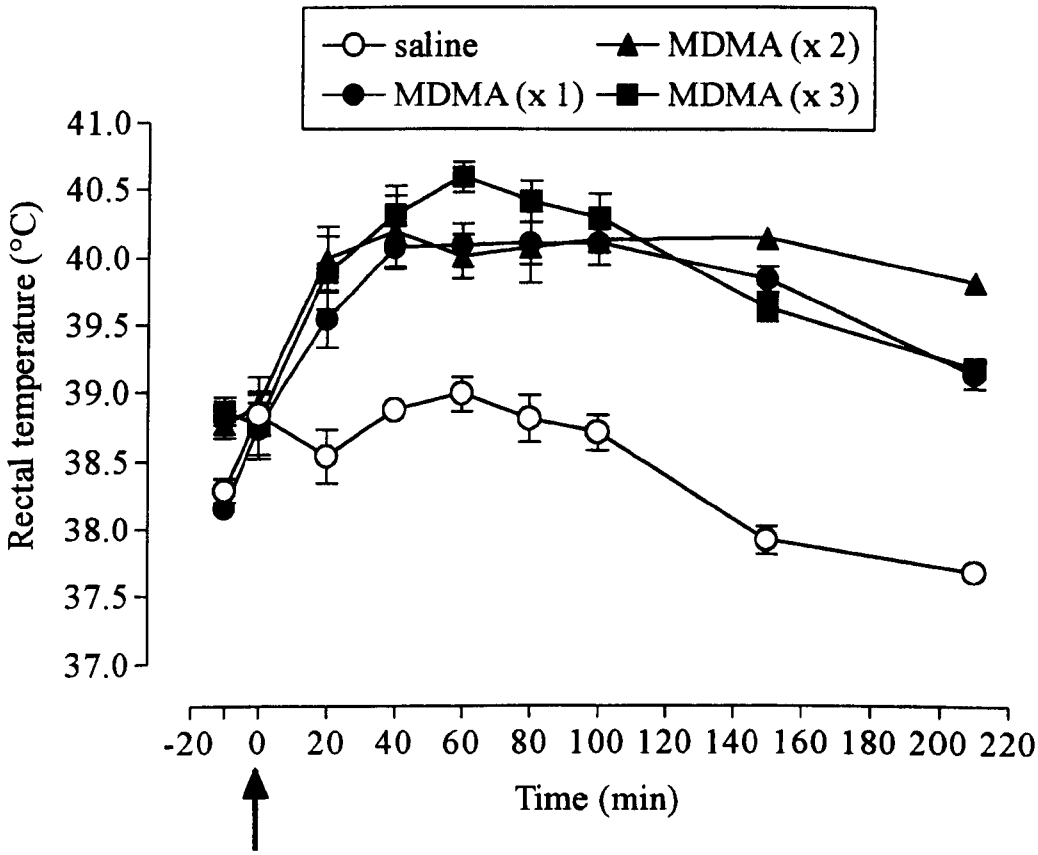

Figure 5.5 Acute effect of MDMA administration (1, 2 or 3 doses) on rectal temperature.

Rats were administered MDMA (12.5 mg/kg i.p.) or saline (denoted by arrow) on 3 separate occasions: Day 1, Day 24 and Day 36. Results shown as mean ± s.e.m., n = 5 - 6 in each group. There was no difference in the basal temperature of the groups. The first injection of MDMA produced a significant rise in rectal temperature compared to saline-treated control animals ($F(1, 10) = 100.4$, $p < 0.001$). A hyperthermic response was also evident after the second ($F(1, 10) = 104.3$, $p < 0.001$) and third ($F(1, 9) = 115.5$, $p < 0.001$) MDMA injection. There was no difference in hyperthermic response between the 3 MDMA treatment groups.

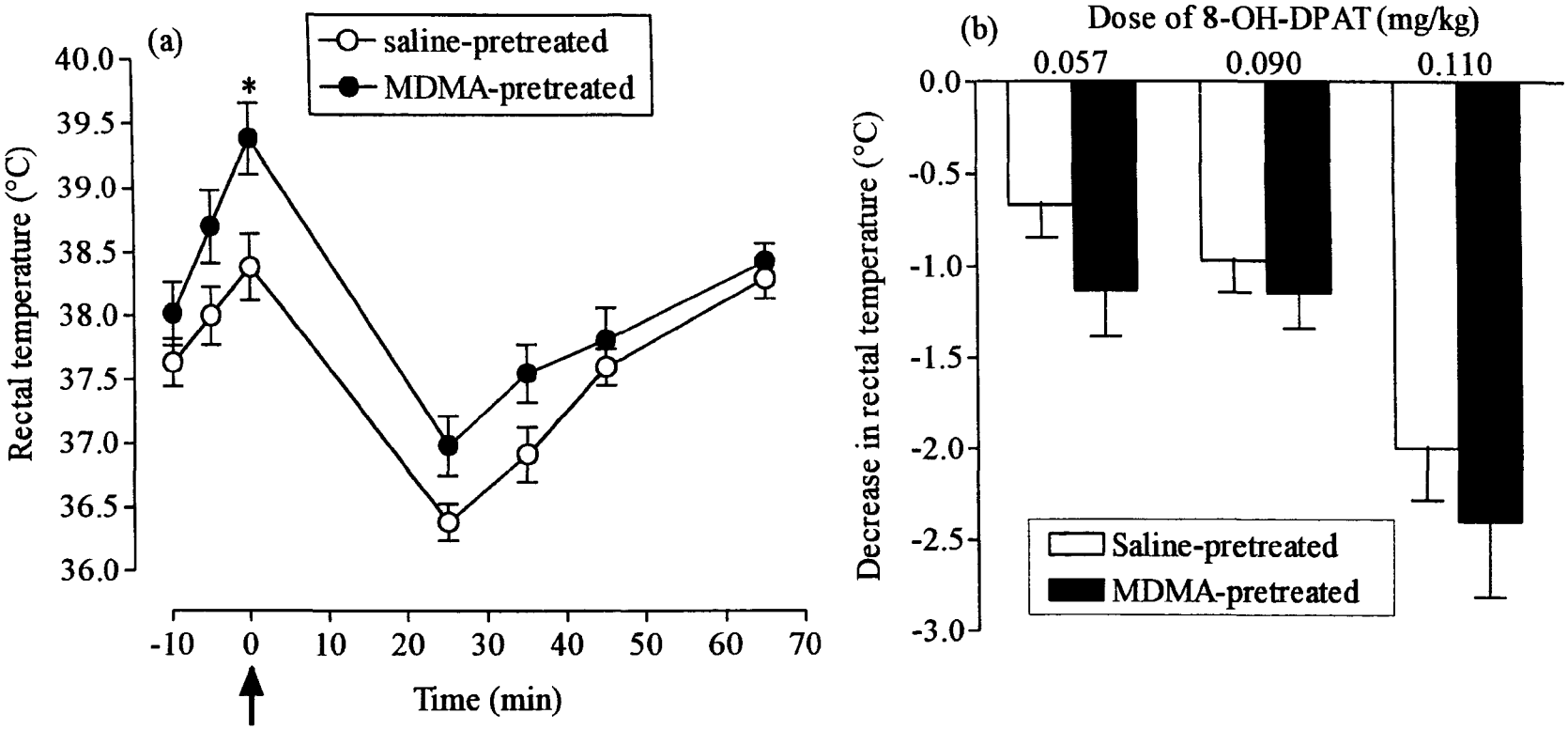

Figure 5.6 Effect of 8-OH-DPAT on the rectal temperature of rats 3 - 4 weeks following administration of MDMA.

Rats were administered 8-OH-DPAT (denoted by arrow) 3 - 4 weeks after administration of a single dose of MDMA (12.5 mg/kg i.p.) or saline. Results shown as mean ± s.e.m., n = 6 in each group. ***(a) Rectal temperature response following administration of 8-OH-DPAT (0.11 mg/kg s.c.).*** MDMA-pretreated animals were different from control animals ($F(1, 25) = 17.0$, $p < 0.001$) and post-hoc analysis demonstrated a statistically significant difference at t = 0 min. Rectal temperature differed over time ($F(4, 25) = 30.8$, $p < 0.0001$). ***(b) Rectal temperature response following administration of 8-OH-DPAT (0.057, 0.090, 0.110 mg/kg s.c.).*** Results shown as change in temperature, 25 min post-injection. MDMA-pretreated animals were not different from control animals at any of the 3 doses of 8-OH-DPAT.

Drug	Dose (mg/kg)	Rectal temperature (°C) t = 0 min	t = 60 min	ΔT
DOI	1.0	39.4 ± 0.1	38.4 ± 0.3	-1.0
	2.0	39.2 ± 0.1	38.7 ± 0.2	-0.5
m-CPP	1.5	39.3 ± 0.2	39.2 ± 0.1	-0.1
	2.5	39.2 ± 0.1	38.9 ± 0.1	-0.3
	5.0	38.5 ± 0.5	38.6 ± 0.1	+0.1
	10.0	39.3 ± 0.1	38.2 ± 0.2	-1.1

Table 5.2 Acute effect of administration of DOI and *m*-CPP on rectal temperature.

Rats were administered DOI (1 or 2 mg/kg i.p.) or *m*-CPP (1.5, 2.5, 5.0 or 10.0 mg/kg i.p.). Results shown as mean ± s.e.m., n = 4 in each group. Change in temperature (ΔT) was calculated as the difference in rectal temperature between t = 0 and t = 60 min.

5.4 DISCUSSION

This study has demonstrated that a single neurotoxic dose of MDMA (12.5 mg/kg) can have longer-term effects on thermoregulation in rats, when exposed to a high ambient temperature "thermoregulatory challenge". In addition, three doses of MDMA administered several weeks apart resulted in a pronounced acute hyperthermic response on each occasion, even though regional brain concentrations of 5-HT were depleted by 20 - 40 % one week after a single dose (12.5 mg/kg).

In concurrence with previous studies (e.g. Broening *et al.*, 1995; Colado *et al.*, 1993; Dafters, 1994; 1995; Nash *et al.*, 1988; O'Shea *et al.*, 1998), an acute hyperthermic response was seen following MDMA administration. A single dose of MDMA (12.5 mg/kg i.p.) resulted in a rectal temperature rise of approximately 1.5 - 2 °C, which peaked within 60 min of drug administration and was sustained for over 3.5 h. Rectal temperature had returned to control values within 24 h and there was no difference between MDMA- and saline-treated animals, with respect to this measure, over the subsequent two weeks. Overall, MDMA treatment did not have any significant effect on weight gain - although the MDMA treatment group was of lower weight than the control group throughout the measurement period, when post-treatment weight was calculated as a % of pretreatment values there was no difference between the treatment groups. However, a transient decrease in weight was observed in the MDMA treatment group on the day following drug administration, indicative of an anorexic effect, which is consistent with previous studies (see Colado *et al.*, 1997b).

One week after administration of MDMA (12.5 mg/kg i.p.), cortical, hippocampal, hypothalamic and striatal concentrations of 5-HT and 5-HIAA were analysed using h.p.l.c. The extent of 5-HT depletion ranged from 21 % in the cortex and striatum, to 32 % and 41 % in the hippocampus and hypothalamus, respectively. 5-HIAA was also depleted in all four brain regions (16 - 35 %). The extent of damage to serotonergic systems seen in the current study, as indicated by depletion of 5-HT and its metabolite, is also in agreement with previous studies in rats (e.g. Aguirre *et al.*, 1998; Broening *et al.*, 1995; Colado *et al.*, 1993; 1995; 1997a; 1998; Farfel & Seiden, 1995a; Malberg *et al.*, 1996; McNamara *et al.*, 1995; O'Shea *et al.*, 1998; Shankaran & Gudelsky, 1999)

and with reported serotonergic neurotoxicity in humans (e.g. Kish *et al.*, 2000; McCann *et al.*, 1998; Semple *et al.*, 1999).

Five to six weeks after administration of a single dose of MDMA, there was no difference in the rectal temperature of MDMA- and saline-pretreated rats. However, when all animals were exposed to a high T_a environment (30 ± 0.5 °C) for 60 min, the rectal temperature response of MDMA-pretreated rats was markedly different to that of saline-pretreated animals. Rats which had been administered MDMA several weeks earlier demonstrated a faster rise in rectal temperature during the "thermoregulatory challenge"; this group reached a peak hyperthermic response of approximately 0.7 °C, 40 min after commencement of the challenge, which was significantly greater than the response of the saline-pretreated control group at this time-point. The rectal temperature of the control group increased steadily during the challenge, reached a maximal hyperthermic response of approximately 0.8 °C by the end of the challenge, and then declined steadily towards pre-challenge values. However, when MDMA-pretreated animals were returned to a 'normal' T_a environment (20 ± 0.5 °C), rectal temperature initially dropped by approximately 0.3 °C, being parallel to the saline-pretreated group during the period t_{60-100}. Subsequently, the rectal temperature of the MDMA-pretreated group rose again and remained approximately 0.5 °C above that of the control group for the remainder of the experiment.

Thus, although MDMA administration had no effect on rectal temperature measured under 'normal' T_a conditions, when MDMA-pretreated animals were subjected to a high T_a "thermoregulatory challenge" an apparent long-term defect in thermoregulation was observed; the hyperthermic response induced by exposure to a high T_a environment was sustained for a significantly longer period of time, compared to control animals. These data are in agreement with those reported by Dafters & Lynch (1998), where MDMA administration resulted in sustained hyperthermic responses to "thermoregulatory challenges" four and 14 weeks post-treatment. It should be noted that rats were group-housed in the current study, which might be expected to affect their thermoregulatory responses. The sustained hyperthermia observed in MDMA-treated rats could involve a behavioural response, whereby group-housed rats huddle together, rather than a

physiological response resulting from MDMA administration. However, animals were housed individually in the study performed by Dafters & Lynch (1998), indicating that the sustained hyperthermia was a genuine physiological response. Furthermore, the current study has demonstrated that such changes in thermoregulatory mechanisms have resulted from a 20 - 40 % depletion of regional brain 5-HT levels.

In addition to the high T_a challenge, as reported above and by Dafters & Lynch (1998), a second "thermoregulatory challenge" was undertaken: animals were exposed to a low T_a environment (10 ± 0.5 °C) for 60 min. Prior to commencement of the challenge, there was no difference in the rectal temperatures of the MDMA- and saline-pretreated groups. However, between the first and second temperature measurements (t = -10 and t = 0 min, respectively), both groups demonstrated a temperature rise of approximately 1 °C, which is likely to be a response to handling of the animals (see Eikelboom, 1986). Subsequently, both during and after the challenge, both groups demonstrated a steady decline in rectal temperature towards pre-challenge values. The temperature of the MDMA-pretreated group remained higher than that of the control group throughout the challenge ($t_{0 - 60}$) and post-challenge ($t_{60 - 180}$) periods, reaching statistical significance at two time-points (t = 100 and t = 140 min). However, unlike the sustained hyperthermia seen after a high T_a challenge, the pattern of response in this experiment was the same in both MDMA- and saline-pretreated animals.

Therefore, in animals where a long-term defect in thermoregulation is indicated (as seen in the high T_a challenge experiment) perhaps heat-dissipating mechanisms, such as sweating and increasing blood flow to the tail, are compromised. In contrast, the response seen during the low T_a challenge experiment indicates that heat-retaining mechanisms, such as piloerection, are unaffected. However, the lack of difference in temperature response between treatment groups when exposed to low T_a could be due to the fact that animals were group-housed (three per cage). By huddling together, any heat loss would be minimised thus different results might be seen if animals were housed individually. Gordon *et al.* (1991) demonstrated that, during the acute hyperthermic response to MDMA, piloerection occurred and blood flow to the tail (as indicated by measurement of tail skin temperature) was not increased. These responses have been

observed during the work undertaken for this thesis and the lack of significant change in tail temperature following MDMA administration (see Chapter 6) is consistent with Gordon *et al.* (1991). Both piloerection and the lack of increase in blood flow to the tail would be likely to exacerbate hyperthermia by attenuating heat dissipation (Gordon *et al.*, 1991). Thus, it may be that MDMA administration compromises thermoregulatory mechanisms both acutely and when animals are exposed to an environment which "challenges" such mechanisms.

When rats were administered three doses of MDMA, separated by two or three weeks, the acute hyperthermic response to each dose was the same as that seen following previous doses. Thus, it appears that up to 40 % depletion of 5-HT has no long-lasting effect on MDMA-induced acute hyperthermia. Shankaran & Gudelsky (1999) performed a similar study, whereby a neurotoxic MDMA treatment regimen (10 mg/kg i.p., four times at 2 h intervals) resulted in a 45 % reduction of striatal 5-HT, one week later. These authors also demonstrated that, apart from after the highest dose employed (20 mg/kg), MDMA-induced hyperthermia was significantly attenuated in rats which had previously been administered MDMA. However, it is difficult to draw direct comparisons between the current study and the results of Shankaran & Gudelsky (1999), as the latter involved: (1) a different strain of rat (SD), (2) a multiple dose treatment regimen (10 mg/kg, four times at 2 h intervals), and (3) a one week interval between the initial administration of MDMA and a subsequent, single, "challenge" dose. As the extent of 5-HT depletion (one week post-treatment) was similar between the two studies, the differences in strain and treatment regimen appear to have no bearing on the observed differences in results. Also, the striatal depletion of 5-HT reported by Shankaran & Gudelsky (1999) one week post-treatment was similar to that reported by Scanzello *et al.* (1993) two- and eight- weeks post-treatment, where the strain and treatment regimen were identical. Therefore, there is no reason to suppose that the different interval between the first and second doses of MDMA in the current study (three weeks), compared to the Shankaran & Gudelsky (1999) study (one week), had any influence on the differences seen in acute hyperthermic responses to the second dose of MDMA. Since the highest "challenge" dose of MDMA (20 mg/kg) used by Shankaran & Gudelsky (1999) did not significantly attenuate the MDMA-induced acute

hyperthermic response, while the two lower doses did, perhaps there is some threshold dose above which depletion of 5-HT does not affect the temperature response. The 20 - 40 % reduction in regional brain 5-HT concentrations observed in the current study, following a single dose of MDMA (12.5 mg/kg), may have been insufficient to significantly alter MDMA-induced hyperthermia; perhaps only a far more severe loss of 5-HT (70 - 80 %) would have long-lasting functional consequences.

Administration of 8-OH-DPAT three or four weeks after a single neurotoxic dose of MDMA (12.5 mg/kg) resulted in a hypothermic response, which paralleled that of saline-pretreated animals. Although the rectal temperature of the MDMA-pretreated rats was higher than that of saline-pretreated animals throughout the experiment, this difference only reached statistical significance at t = 0 (immediately prior to 8-OH-DPAT administration). There was no difference in the maximal decrease in temperature (at t = 25 min) between MDMA- and saline-pretreated animals, following administration of any of the three doses of 8-OH-DPAT (0.057, 0.09 and 0.11 mg/kg). These results are in agreement with McNamara *et al.* (1995) and indicate that MDMA administration has no long-lasting effect on 5-HT_{1A} receptor function.

However, both the current data and that of McNamara *et al.* (1995) are in contrast to Aguirre *et al.* (1998), who reported that MDMA pretreatment led to a potentiation of 8-OH-DPAT-induced hypothermia. However, Aguirre *et al.* (1998) demonstrated no difference in the 8-OH-DPAT-induced hypothermic response between animals which had received eight doses of MDMA (30 mg/kg i.p., twice daily on four consecutive days), and those which had been administered a single dose (30 mg/kg i.p.). The extent of 5-HT depletion following a multiple dose treatment regimen (Aguirre *et al.*, 1998) was similar to that seen in the current study after a single dose of MDMA. Therefore it would be expected that, if the modification of the 8-OH-DPAT-induced hypothermia was due to 5-HT depletion, then the response in animals which had received a single dose would be different from that in animals administered multiple doses. One possible reason for the discrepancy in results, may be that the 8-OH-DPAT-induced hypothermic response of control animals (approximately -1.8 °C) reported by Aguirre *et al.* (1998) was actually smaller than that seen in other studies (-2.1 to -3 °C: see Goodwin *et al.*,

1987; McNamara *et al.*, 1995; current study). Thus, if 8-OH-DPAT administration had resulted in an acute hypothermia of at least -2 °C, the effect of MDMA pretreatment reported by Aguirre *et al.* (1998) might not have been significant.

Neither DOI nor *m*-CPP had any effect on rectal temperature at the range of doses used in the current study. This is in contrast to the acute hyperthermic responses reported by Mazzola-Pomietto *et al.* (1995; 1996) and Salmi & Ahlenius (1998). Therefore the proposed experiment, whereby MDMA-pretreated animals would have been "challenged" with DOI and *m*-CPP, was not performed. It may be that DA rats are more resistant to any effects of DOI and *m*-CPP, although this seems unlikely since the range of doses used far exceeded those employed by Mazzola-Pomietto *et al.* (1995; 1996) and Salmi & Ahlenius (1998). Since Kennett (personal communication) was also unable to demonstrate consistent temperature changes following administration of DOI or *m*-CPP to SD or Wistar rats, it is possible that acute hyperthermic responses to DOI or *m*-CPP are weak and require particular environmental conditions for their manifestation.

In conclusion, the current study has clearly demonstrated that a single dose of MDMA can result in a 20 - 40 % depletion of regional brain 5-HT and 5-HIAA, one week post-treatment. Since 5-HT is believed to play an important role in temperature regulation (see Kruk & Pycock, 1991; Lucki, 1998; Milton, 1977; Myers, 1981; Rang *et al.*, 1995), it seems that this degree of 5-HT depletion has long-term functional consequences; when rats were exposed to a "thermoregulatory challenge" a sustained hyperthermia was seen, indicating that heat-dissipating mechanisms might be compromised under high T_a conditions. However, a 20 - 40 % loss of 5-HT was not sufficient to modify acute hyperthermic responses to subsequent doses of MDMA. Since heat-dissipating mechanisms also appear to be compromised during an acute hyperthermic response to MDMA (see Gordon *et al.*, 1991; Mechan *et al.*, 2001a), perhaps a far greater 5-HT depletion (70 - 80 %) would be required to have any significant effect on the different mechanisms involved in MDMA-induced acute hyperthermia.

Serotonergic neurotoxicity has also been reported in human recreational users of MDMA, including significant reductions in brain 5-HT content and 5-HT transporter

activity (e.g. Kish *et al.*, 2000; McCann *et al.*, 1998; Semple *et al.*, 1999). One of the major consequences of MDMA ingestion by humans is hyperthermia, with temperatures of over 43 °C being reported (e.g. Chadwick *et al.*, 1991; Randall, 1992; Screaton *et al.*, 1992). This hyperthermic response is believed to be associated with the major adverse clinical effects of MDMA (see Green *et al.*, 1995), such as disseminated intravascular coagulation and rhabdomyolysis (see Chadwick *et al.*, 1991; Henry *et al.*, 1992; Screaton *et al.*, 1992). MDMA administration to rats under high T_a conditions tends to induce hyperthermia, while low T_a conditions tend to induce hypothermia (see Broening *et al.*, 1995; Dafters, 1994; 1995; Dafters & Lynch, 1998; Gordon *et al.*, 1991). Therefore, hyperthermia is likely to occur in human MDMA users, since ingestion usually occurs in hot, crowded environments (see Green *et al.*, 1995; Henry *et al.*, 1992), which may further potentiate the hyperthermic response (Henry *et al.*, 1992).

If brain levels of 5-HT in human recreational users of MDMA are depleted by a similar extent to that seen in rats in the current study, there may be serious functional consequences. Continued use of MDMA under high T_a conditions may significantly compromise thermoregulatory mechanisms, resulting in sustained hyperthermia and additional potentially life-threatening complications.

CHAPTER 6

TEMPERATURE II:

A STUDY OF THE ACUTE HYPERTHERMIC RESPONSE FOLLOWING MDMA ADMINISTRATION TO DARK AGOUTI RATS

6 TEMPERATURE II: A study of the acute hyperthermic response following MDMA administration to Dark Agouti rats

6.1 INTRODUCTION

MDMA administration to rodents and humans results in an acute hyperthermic response which has been well-documented (see Chapter 1; Broening *et al.*, 1995; Chadwick *et al.*, 1991; Colado *et al.*, 1993; Dafters, 1994; 1995; Nash *et al.*, 1988; O'Shea *et al.*, 1998; Randall, 1992; Schmidt *et al.*, 1990a; Screaton *et al.*, 1992). However, the specific pharmacology of this response has not been clarified, thus the current chapter comprises an investigation of MDMA-induced acute hyperthermia in rats, through the use of a series of drugs which affect serotonergic or dopaminergic function.

6.1.1 Acute hyperthermic response following MDMA administration: involvement of 5-HT

Grahame-Smith (1971) demonstrated that administration of a monoamine oxidase (MAO) inhibitor (tranylcypromine (TCP); 20 mg/kg i.p.) 30 min prior to L-tryptophan (30 - 200 mg/kg i.p.), resulted in a dose-dependent increase in 5-HT synthesis within the brain. Acute hyperactivity and hyperthermia were observed and peaked within 30 min and 60 min after L-tryptophan injection, respectively, and these responses were seen to positively correlate with the accumulation of brain 5-HT (Grahame-Smith, 1971). MDMA administration results in a substantial, acute release of 5-HT from serotonergic nerve endings (see Colado & Green, 1994; Crespi *et al.*, 1997; Johnson *et al.*, 1986; Koch & Galloway, 1997; Mechan *et al.*, 2000; Sabol & Seiden, 1998; Schmidt *et al.*, 1987; Schmidt & Taylor, 1988; Shankaran & Gudelsky, 1999; Stone *et al.*, 1986; 1987b), while the acute hyperthermic response, which also results from MDMA treatment, has been postulated to be a consequence of 5-HT release and the subsequent stimulation of 5-HT_2 receptors (see Shankaran & Gudelsky, 1999). This suggestion is further supported by the fact that: (1) other 5-HT releasing drugs, such as *p*-chloroamphetamine (PCA) and fenfluramine (see Berger *et al.*, 1992; Crespi *et al.*, 1997; Martín & Artigas, 1992), also produce hyperthermia (Colado *et al.*, 1993; Pawlowski, 1981; Sugrue, 1984), and (2) 5-HT_2 receptor antagonists, such as ketanserin

and MDL 11,939, block the MDMA-induced acute hyperthermic response (Malberg *et al.*, 1996; Nash *et al.*, 1988; Schmidt *et al.*, 1990a).

6.1.2 Aims of the investigation: the use of drugs affecting 5-HT or dopamine function

The current study was undertaken in an attempt to elucidate the mechanisms involved in MDMA-induced acute hyperthermia in rats, and a series of drugs which affect 5-HT or dopamine function were employed for this purpose (see below). Rectal temperature was closely monitored during each experiment, as it was important that each pretreatment compound under investigation did not induce hypothermia in control animals. (If hypothermia was seen in control rats, any effect on MDMA-induced hyperthermia could not then be easily attributed to MDMA-mediated events, but could be due to effects of the pretreatment compound alone).

6.1.2.1 5-$HT_{1/2}$ and 5-HT_2 receptor antagonists

- Methysergide, a non-selective 5-$HT_{1/2}$ receptor antagonist, which has been demonstrated to block fenfluramine-induced acute hyperthermia (Sugrue, 1984).
- MDL 11,939, a 5-HT_2 antagonist, which has previously been reported to competitively antagonise MDMA-induced hyperthermia in male SD rats: Schmidt *et al.* (1990a) co-administered MDL 11,939 (5 mg/kg s.c.) with MDMA (10, 20 and 30 mg/kg s.c.), and demonstrated a complete blockade of MDMA-induced hyperthermia at the lowest dose and significant attenuation at the two higher doses. MDL 11,939 also attenuated MDMA-induced reductions in brain 5-HT concentration, measured one week later (Schmidt *et al.*, 1990a; 1990b; 1990c).
- Ritanserin, a 5-HT_2 antagonist, which has been demonstrated to prevent the acute hyperthermia induced by co-administration of an MAO inhibitor (clorgyline) and 5-hydroxy-L-tryptophan (5-HTP) (Nisijima *et al.*, 2001), and to attenuate MDMA-induced 5-HT depletion (Schmidt *et al.*, 1990b).

6.1.2.2 5-HT_{2A} and 5-HT_{2C} receptor antagonists

- MDL 100,907, a selective 5-HT_{2A} antagonist, which has been shown to prevent MDMA-induced reductions in regional brain 5-HT concentrations and MDMA-induced stimulation of striatal dopamine synthesis (Schmidt *et al.*, 1992)
- SB 242084, a selective 5-HT_{2C} antagonist, which potently inhibits *m*-CPP-induced hypolocomotion, an *in vivo* model of central 5-HT_{2C} receptor function (Kennett *et al.*, 1997).

6.1.2.3 Serotonin reuptake inhibitors

- Zimeldine, which has been reported to have no effect on fenfluramine-induced acute hyperthermia (Sugrue, 1984).
- Fluoxetine, which has been demonstrated to significantly attenuate the synaptosomal release of [^{3}H]5-HT induced by MDMA, PCA, fenfluramine and methamphetamine (Berger *et al.*, 1992) and to attenuate fenfluramine-induced hyperthermia (Sugrue, 1984).

6.1.2.4 Dopamine D_1 and D_2 receptor antagonists

- Remoxipride, a dopamine D_2 receptor antagonist.
- SCH 23390, a dopamine D_1 antagonist, which has been reported to attenuate both methamphetamine-induced hyperthermia and synaptosomal [^{3}H]dopamine uptake (Metzger *et al.*, 2000).

6.1.2.5 Dopamine reuptake inhibitors

- GBR 12909, which has been demonstrated to significantly attenuate MDMA-induced deficits in striatal 5-HT and 5-HIAA content and tryptophan hydroxylase (TPH) activity (Stone *et al.*, 1988), in addition to attenuating MDMA-induced increases in extracellular dopamine concentrations, as measured via *in vivo* microdialysis (Nash & Brodkin, 1991).

6.2 METHODS

6.2.1 Animals, drug administration and temperature measurement

Male DA rats were housed as detailed in sections 2.1(1) and 2.1(3). All pretreatment compounds were injected 20 min prior to injection of MDMA (12.5 mg/kg i.p.) or vehicle, with the exception of the fluoxetine experiment where fluoxetine was administered 5 min before and 55 min after administration of MDMA (15 mg/kg i.p.) or vehicle. (Note that the latter experiment was undertaken by the research group of Dr M.I. Colado and, as such, the protocol employed was that in common use in their laboratory).

All drugs were dissolved in normal saline and injected i.p. unless otherwise stated and doses are quoted as the base weight. Methysergide was dissolved in 20 % DMSO in normal saline. Ritanserin, MDL 100,907 and MDL 11,939 were dissolved in 2 % glacial acetic acid in deionised water. SB 242084 was dissolved in normal saline and citric acid (25 mM) containing a saturated solution of β-cyclodextrin and 30 % polyethylene glycol. GBR 12909 was dissolved in peanut oil and administered s.c. in a volume of 2 ml/kg.

Rectal temperature was measured, as detailed in section 2.7, at regular intervals during all experiments. During one experiment, tail skin temperature was also measured by binding the temperature probe to the upper tail region with plastic tape.

6.2.2 Statistics

Statistical analysis of acute rectal and tail skin temperature data were performed using GraphPad Prism. Data were analysed by two-way ANOVA with repeated measures, with TREATMENT (MDMA or vehicle) as the between subjects factor and TIME as the repeated measure.

Statistical analyses of the antagonist/uptake inhibitor pretreatment studies and microdialysis experiments were performed using BMDP/386 Dynamic. Data were analysed by two-way ANOVA with repeated measures (programme 2V) or, where

missing values occurred, an unbalanced repeated measures model (programme 5V) was used. Both used TREATMENT as the between-subjects factor and TIME as the repeated measure. ANOVA was performed on both pre-treatment and post-treatment (starting immediately after MDMA/saline injection) data.

6.3 RESULTS

6.3.1 Effect of MDMA administration on rectal and tail skin temperature

MDMA administration (12.5 mg/kg i.p.) resulted in a rectal temperature rise of approximately +1.2 °C compared to the control group, which reached a peak 40 min after injection and was sustained for over 3 h (Figure 6.1a). This resulted in a main effect of TREATMENT. Following the initial temperature rise, rectal temperature subsequently declined towards pretreatment values resulting in a main effect of TIME, but no interaction effect.

Tail skin temperature was also measured immediately prior to, and at regular intervals after, administration of MDMA or saline (Figure 6.1b). The initial tail temperatures were: MDMA-treated group, 27.2 ± 1°C; control group: 25.5 ± 0.5 °C. Although the tail temperature of the MDMA-treated group was lower than that of the control group during the period t_{40-80} and at t = 180 min, overall there was no statistically significant difference between the two treatment groups.

6.3.2 Effect of methysergide, MDL 11,939 and ritanserin on MDMA-induced hyperthermia

The non-selective 5-$HT_{1/2}$ receptor antagonist methysergide (10 mg/kg i.p.). did not modify the MDMA-induced hyperthermic response and had no effect on the rectal temperature of control animals (Figure 6.2). The 5-HT_2 antagonist MDL 11,939 (5 mg/kg i.p.) abolished the MDMA-induced hyperthermia (Figure 6.3) without altering the rectal temperature of control animals. However, the 5-HT_2 antagonist ritanserin (1 mg/kg i.p.) had no effect on the MDMA-induced acute hyperthermic response (Figure 6.4) and did not alter the rectal temperature of control animals.

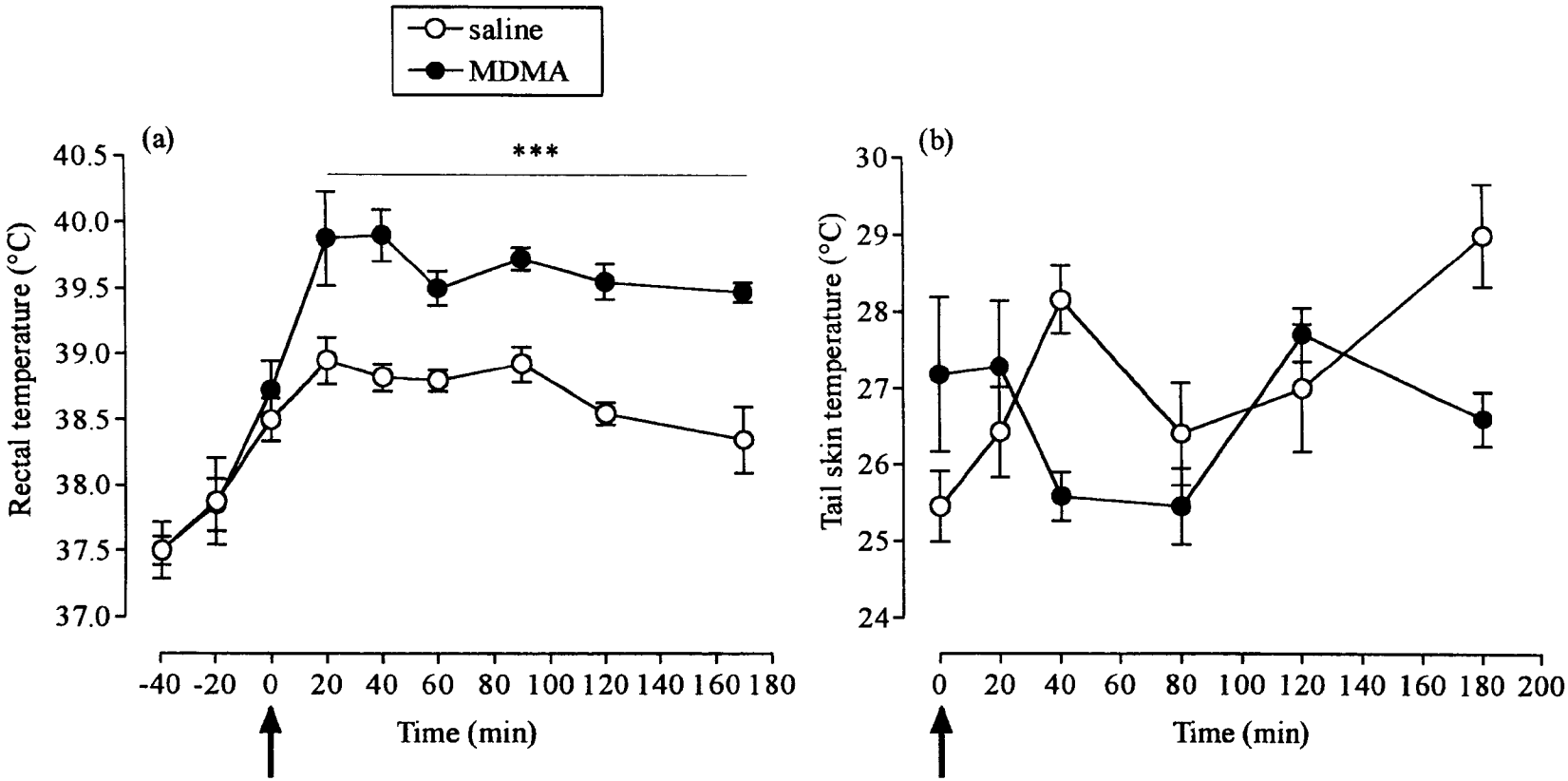

Figure 6.1 Acute effect of MDMA administration on rectal and tail skin temperature.

Rats were administered MDMA (12.5 mg/kg i.p.) or saline (denoted by arrows). Results shown as mean ± s.e.m., n = 3 – 4 in each group. ***(a) Rectal temperature.*** MDMA induced a significant rise in rectal temperature compared to saline-treated control animals ($F(1, 21) = 246.5$, $p < 0.0001$) and post-hoc analysis demonstrated statistically significant differences throughout the period t_{20-180} (***$p < 0.001$). Rectal temperature changed over time ($F(6, 21) = 3.27$, $p < 0.05$). ***(b) Tail skin temperature.*** There was no statistically significant difference between treatment groups.

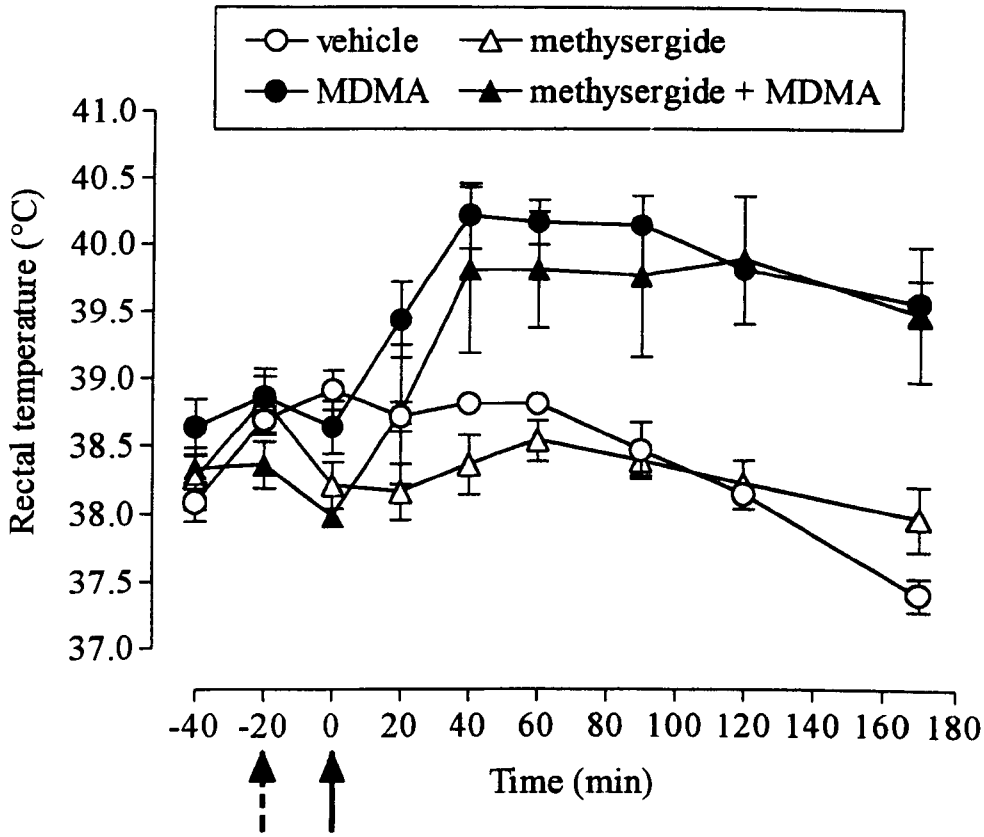

Figure 6.2 Effect of methysergide on MDMA-induced acute hyperthermia.

Rats were administered methysergide (10 mg/kg i.p.) or vehicle (denoted by broken arrow) 20 min prior to injection with MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 4 – 5 in each group. There was no difference in the basal temperatures of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 6) = 76.4$, $p < 0.001$) compared to the vehicle-treated control group. Methysergide did not modify the MDMA-induced hyperthermic response and had no effect on the temperature of control animals.

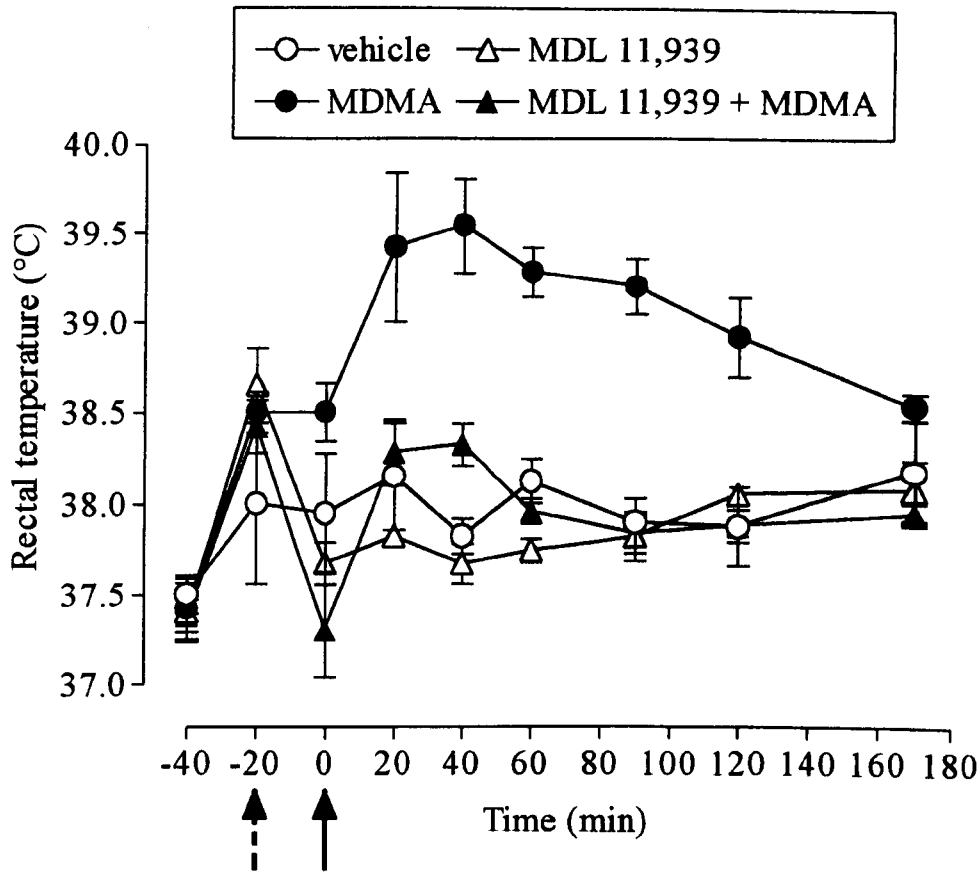

Figure 6.3 Effect of MDL 11,939 on MDMA-induced acute hyperthermia.

Rats were administered MDL 11,939 (5 mg/kg i.p.) or vehicle (denoted by broken arrow) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 4 – 5 in each group. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 7) = 36.3$, $p < 0.001$) compared to the vehicle-treated control group. MDL 11,939 abolished the MDMA-induced hyperthermia ($F(1, 8) = 73.5$, $p < 0.001$) without altering the temperature of control rats.

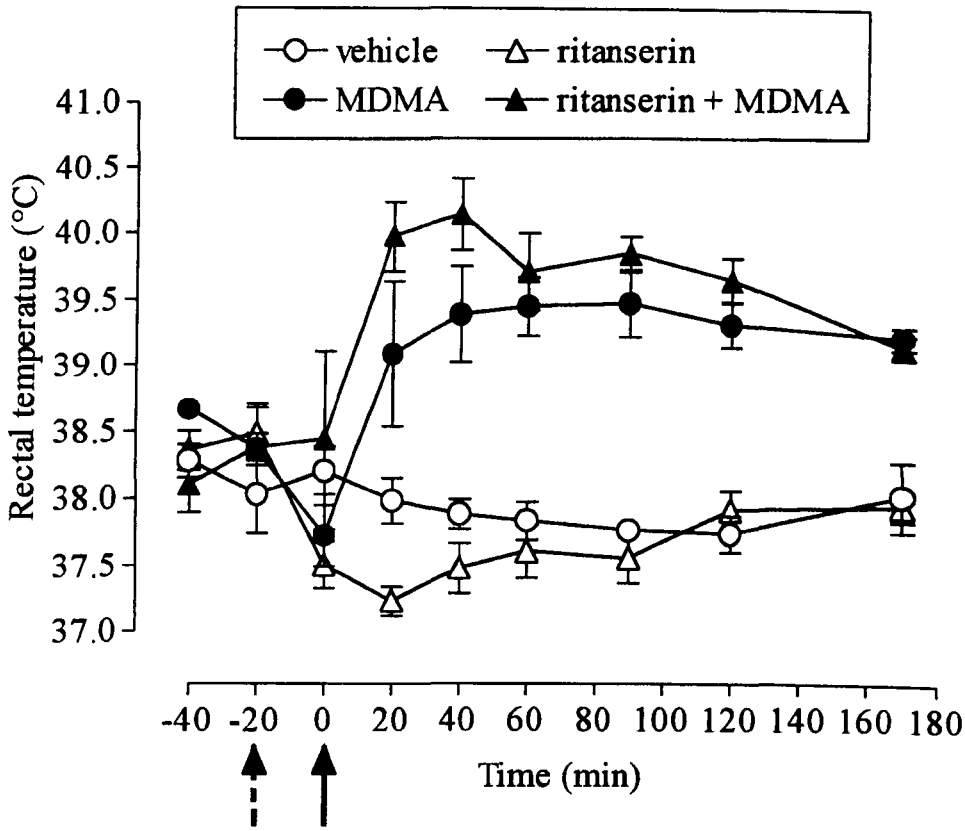

Figure 6.4 Effect of ritanserin on MDMA-induced acute hyperthermia.

Rats were administered ritanserin (1 mg/kg i.p.) or vehicle (denoted by broken arrow) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 4 – 5 in each group. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 7) = 21.0$, $p < 0.01$) compared to the vehicle-treated control group. Ritanserin did not modify the MDMA-induced hyperthermia and had no effect on the rectal temperature of control animals.

6.3.3 Effect of selective 5-HT_{2A} and 5-HT_{2C} receptor antagonists on MDMA-induced hyperthermia

The potent and selective 5-HT_{2A} antagonist MDL 100,907 (0.1 mg/kg i.p.) had no effect on MDMA-induced hyperthermia and did not alter the basal temperature of control animals (Figure 6.5a). Modest attenuation of MDMA-induced hyperthermia was observed at a dose of 0.3 mg/kg i.p., without altering the rectal temperature of control animals (Figure 6.5b). The 5-HT_{2C} antagonist SB 242084 (3 mg/kg i.p.) did not alter MDMA-induced hyperthermia and had no effect on the rectal temperature of control rats (Figure 6.6).

6.3.4 Effect of 5-HT uptake inhibitors on MDMA-induced hyperthermia

The 5-HT uptake inhibitor zimeldine was administered at a dose of 10 mg/kg i.p. (Figure 6.7a), and did not alter MDMA-induced hyperthermia or have any effect on the rectal temperature of control rats. The 5-HT uptake inhibitor fluoxetine (10 mg/kg i.p.) also had no effect on the MDMA-induced hyperthermic response or on the temperature of control rats (Figure 6.7b).

6.3.5 Effect of remoxipride and SCH 23390 on MDMA-induced hyperthermia

The dopamine D_2 receptor-selective antagonist remoxipride (10 mg/kg i.p.) had no effect on the MDMA-induced hyperthermic response (Figure 6.8). However, in contrast, the dopamine D_1 receptor-selective antagonist SCH 23390 (1 and 2 mg/kg i.p.) attenuated the MDMA-induced hyperthermic response, without altering the rectal temperature of control rats (Figure 6.9a and b). This effect was dose-dependent; the difference in rectal temperature between MDMA and SCH 23390 + MDMA treatment groups (as measured at the time of the maximal MDMA-induced hyperthermic response; 90 min) increased with increasing dose of SCH 23390 (0.3, 1 and 2 mg/kg; Figure 6.9c). At the highest dose of SCH 23390 (2.0 mg/kg i.p.), the temperature of the MDMA + SCH 23390 group was significantly lower than that of the control group during t_{20-170} post-MDMA injection (Figure 6.9b).

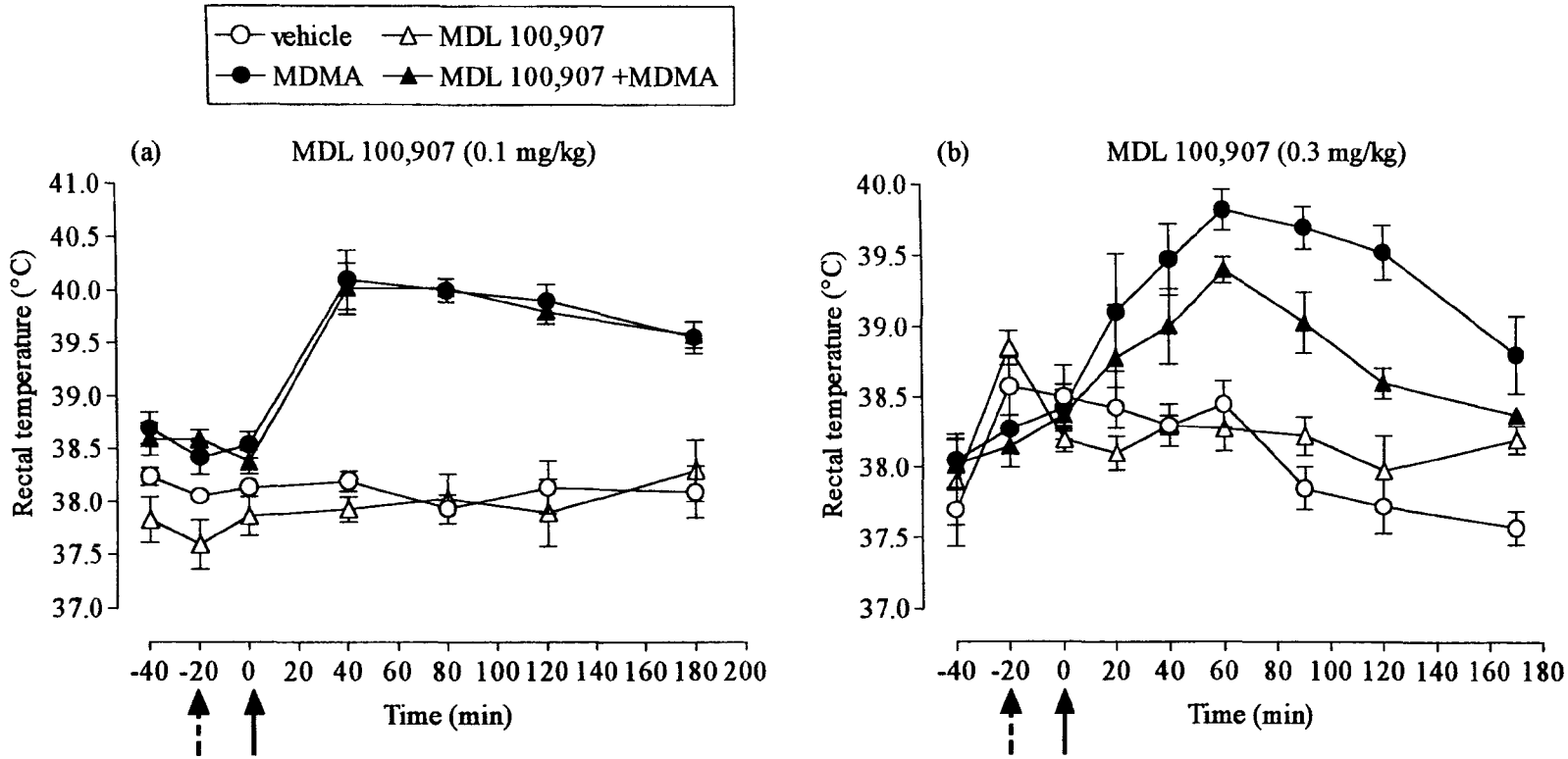

Figure 6.5 Effect of MDL 100,907 on MDMA-induced acute hyperthermia.

Rats were administered MDL 100, 907 (0.1 or 0.3 mg/kg i.p.) or vehicle (denoted by broken arrows) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrows). Results shown as mean ± s.e.m., n = 4 – 5 in each group. ***(a) MDL 100,907 (0.1 mg/kg).*** MDMA produced a significant rise in rectal temperature ($F(1, 8) = 86.0$, $p < 0.001$) compared to the vehicle-treated control group. MDL 100,907 did not modify the MDMA-induced hyperthermia and had no effect on the rectal temperature of control animals. ***(b) MDL 100,907 (0.3 mg/kg).*** MDMA produced a significant rise in rectal temperature ($F(1, 6) = 307.0$, $p < 0.001$) compared to the control group. MDL 100,907 attenuated the MDMA-induced hyperthermia ($F(1, 6) = 22.4$, $p < 0.01$) without altering the rectal temperature of control animals.

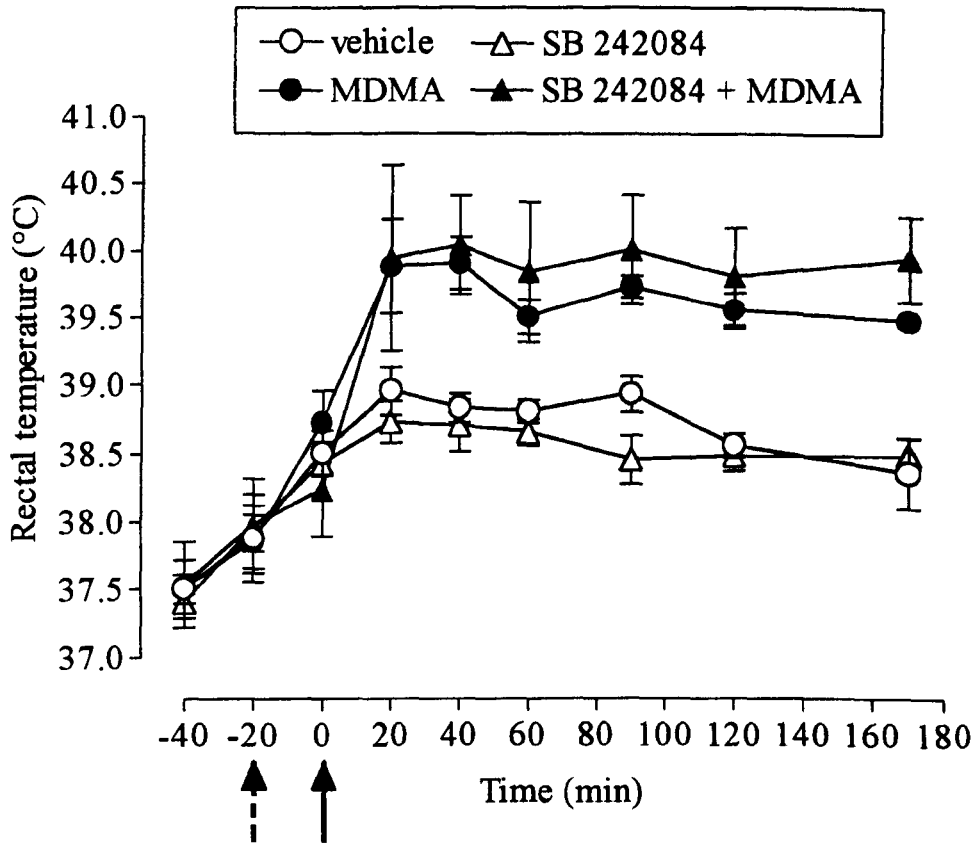

Figure 6.6 Effect of SB 242084 on MDMA-induced acute hyperthermia.

Rats were administered SB 242084 (3 mg/kg i.p.) or vehicle (denoted by broken arrow) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 3 - 4 in each group. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 6) = 70.4$, $p < 0.001$) compared with the vehicle-treated control group. SB 242084 did not modify the MDMA-induced hyperthermic response and had no effect on the rectal temperature of control rats.

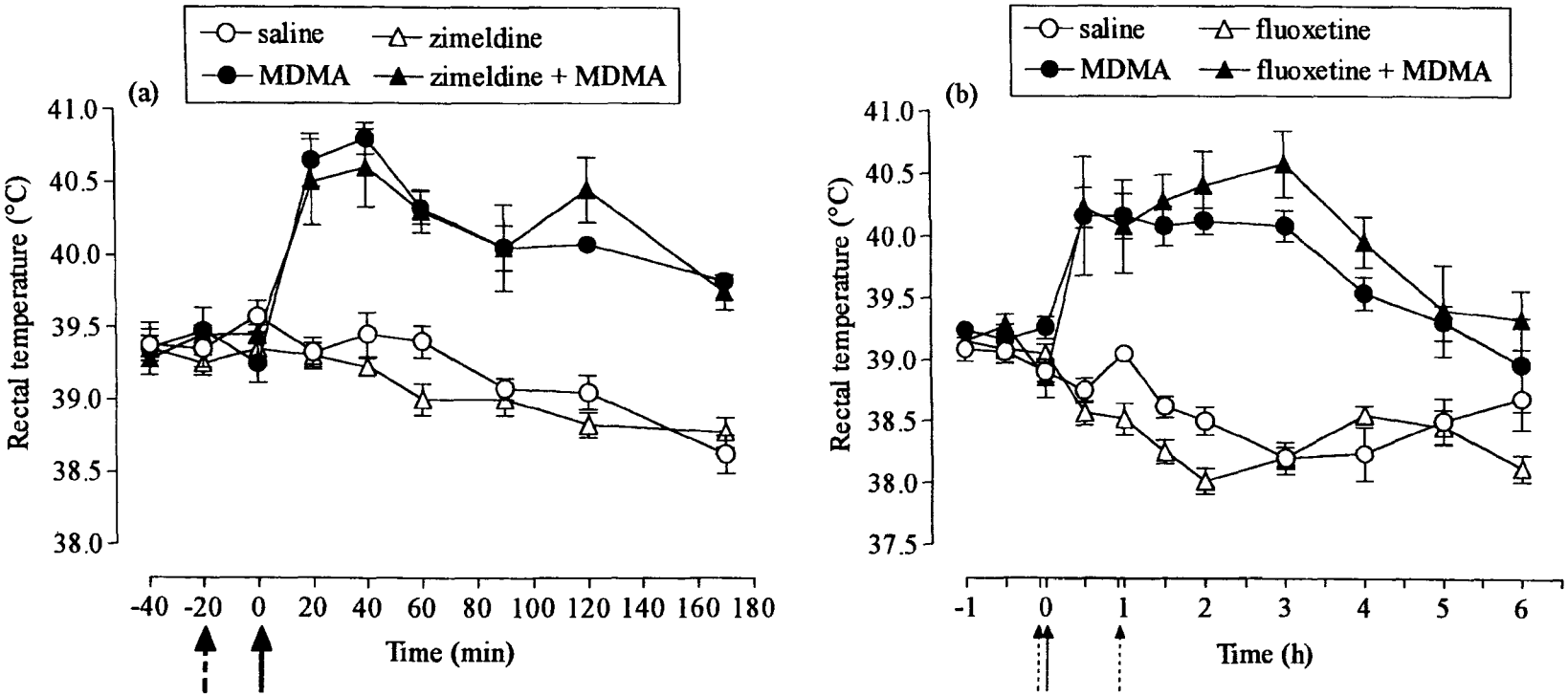

Figure 6.7 Effect of 5-HT uptake inhibitors on MDMA-induced acute hyperthermia.

(a) Zimeldine. Rats were administered zimeldine (10 mg/kg i.p.) or vehicle (denoted by broken arrow) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 4 in each group. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 6) = 65.6$, $p < 0.001$) compared with the vehicle-treated control group. Zimeldine did not modify the MDMA-induced hyperthermic response and had no effect on the rectal temperature of control rats. ***(b) Fluoxetine***. Rats were administered fluoxetine (10 mg/kg i.p.) or vehicle (denoted by broken arrows) 5 min before and 55 min after administration of MDMA (15 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 4 – 6 in each group. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 9) = 74.3$, $p < 0.001$) compared with the vehicle-treated control group. Fluoxetine did not modify the MDMA-induced hyperthermic response and had no effect on the rectal temperature of control rats.

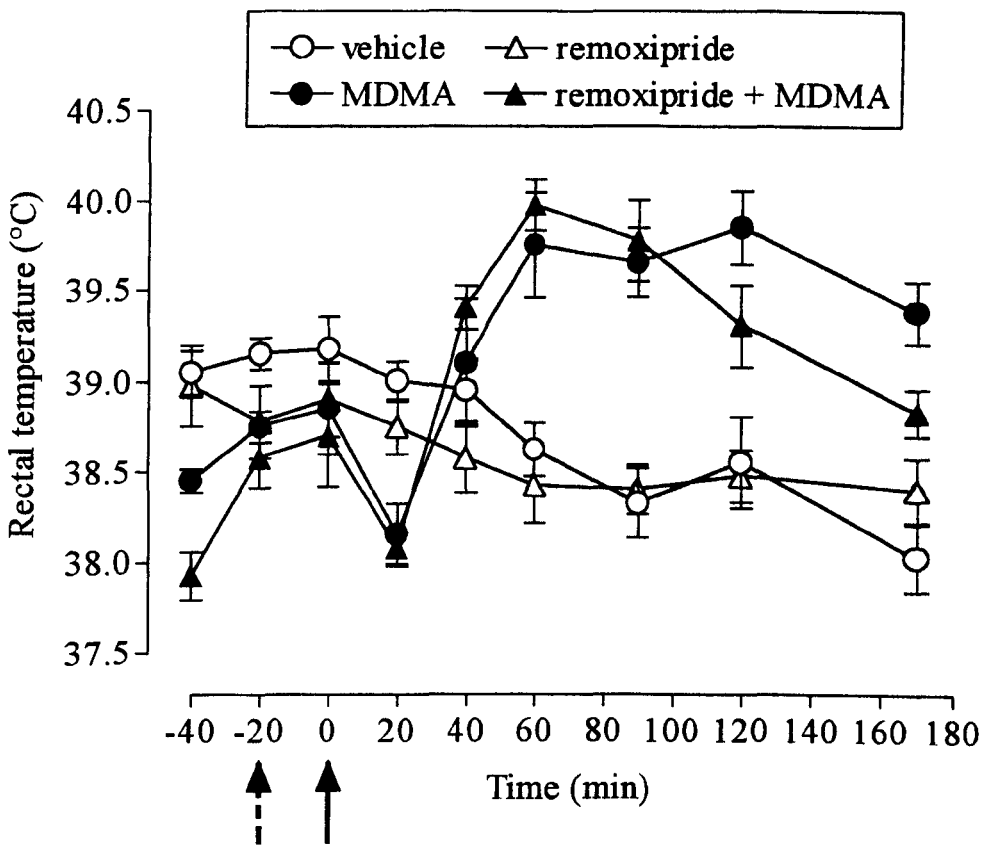

Figure 6.8 Effect of remoxipride on MDMA-induced acute hyperthermia.

Rats were administered remoxipride (10 mg/kg i.p.) or vehicle (denoted by broken arrow) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 3 - 4 in each group. MDMA produced a significant rise in rectal temperature, over 40 min after injection ($F(1, 6) = 15.3$, $p < 0.01$) compared with the vehicle-treated control group. Remoxipride did not modify the MDMA-induced hyperthermic response and had no effect on the rectal temperature of control rats.

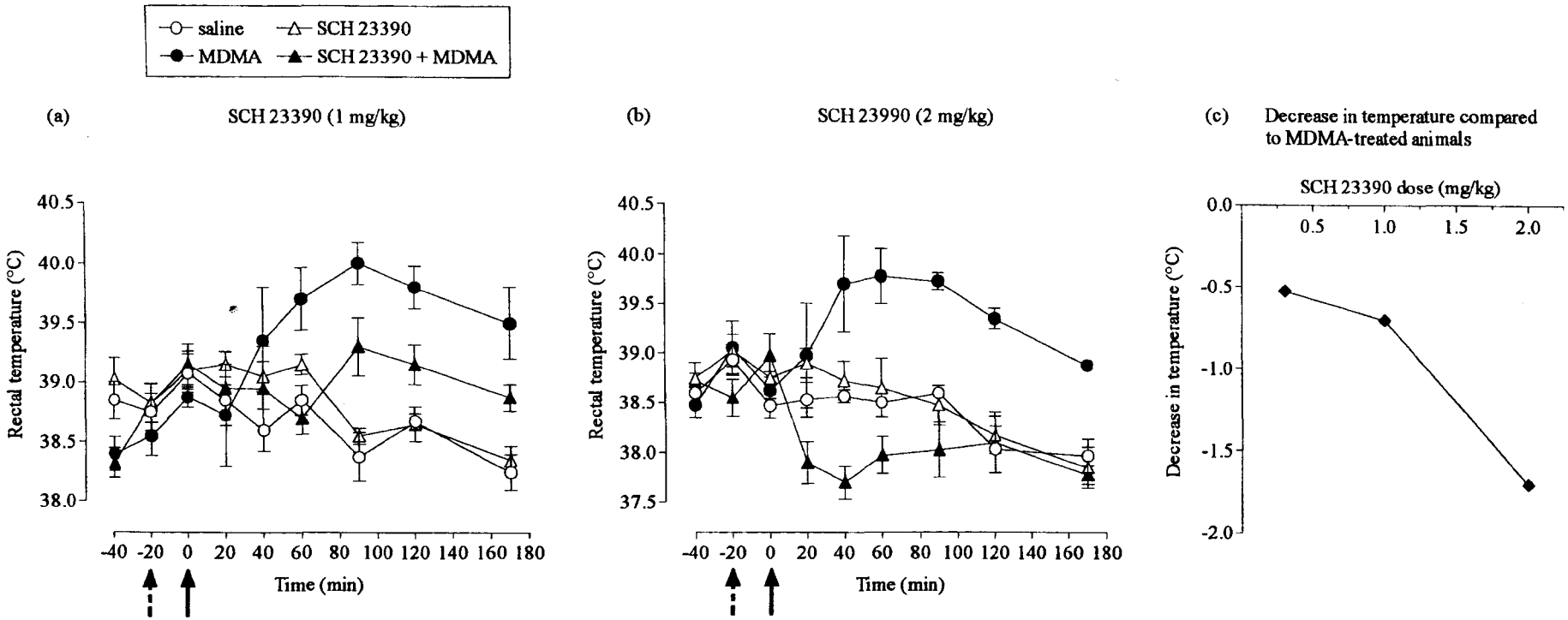

Figure 6.9 Effect of SCH 23390 on MDMA-induced acute hyperthermia.

Rats were administered SCH 23390 (1 or 2 mg/kg i.p.) or vehicle (denoted by broken arrows) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrows). Results shown as mean ± s.e.m., n = 3 - 4 in each group. ***(a) SCH 23390 (1 mg/kg).*** MDMA produced a significant rise in rectal temperature ($F(1, 6) = 8.0$, $p < 0.05$) compared with the vehicle-injected control group. SCH 23390 attenuated the MDMA-induced hyperthermic response ($F(1, 6) = 8.8$, $p < 0.05$) during t_{60-170} without altering the rectal temperature of control rats. ***(b) SCH 23390 (2 mg/kg).*** MDMA produced a significant rise in rectal temperature ($F(1, 5) = 16.4$, $p < 0.01$) compared with the vehicle-treated control group. SCH 23390 abolished the MDMA-induced hyperthermic response ($F(1, 6) = 25.6$, $p < 0.01$) without altering the rectal temperature of control rats. ***(c) Difference in rectal temperature between MDMA and SCH 23390 + MDMA treatment groups.*** Values calculated as the decrease in temperature at 90 min (maximal hyperthermic response in MDMA-treated animals) in the SCH 23390 + MDMA treatment group compared to the MDMA treatment group. Increasing the dose of SCH 23390 resulted in greater decreases in rectal temperature.

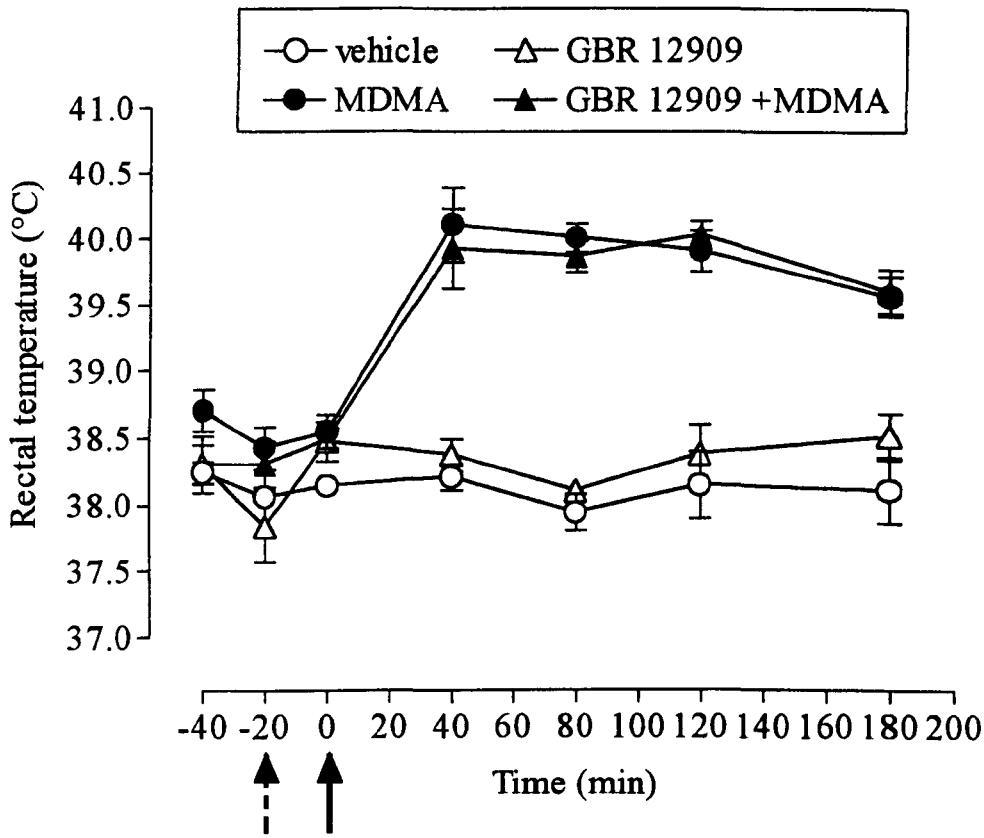

Figure 6.10 Effect of GBR 12909 on MDMA-induced acute hyperthermia.

Rats were administered GBR 12909 (10 mg/kg s.c.) or vehicle (denoted by broken arrow) 20 min before administration of MDMA (12.5 mg/kg i.p.) or saline (denoted by full arrow). Results shown as mean ± s.e.m., n = 3 - 4 in each group. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 8) = 86.0$, $p < 0.001$) compared with the control group. GBR 12909 did not modify the MDMA-induced hyperthermic response and had no effect on the rectal temperature of control rats.

6.3.6 Effect of GBR 12909 on MDMA-induced hyperthermia

The selective dopamine uptake inhibitor GBR 12909 (10 mg/kg s.c.) did not alter the MDMA-induced hyperthermia and had no effect on the rectal temperature of control animals (Figure 6.10).

6.4 DISCUSSION

This study has demonstrated that the acute hyperthermic response which follows administration of MDMA to rats does not appear to be solely mediated by 5-HT receptors, and that dopamine D_1 receptors play a major role in the response.

As reported in previous studies (e.g. Broening *et al.*, 1995; Colado *et al.*, 1993; Dafters, 1994; 1995; Nash *et al.*, 1988; O'Shea *et al.*, 1998), and as demonstrated in Chapter 5, administration of MDMA (12.5 mg/kg i.p.) resulted in an acute hyperthermic response of approximately +1.2 °C. However, the rise in rectal temperature was not accompanied by an overall change in tail skin temperature; while the maximal increase in rectal temperature occurred 40 min post-injection, the tail temperatures of the MDMA-treated group were lower than those of the control group during the period t_{40-80} and at t = 180 min, although this difference was not statistically significant. Tail skin temperature can be used to provide an estimation of tail blood flow (Gordon *et al.*, 1991; Mohaghegh *et al.*, 1997), and has previously been demonstrated not to increase following administration of MDMA (Gordon *et al.*, 1991) or methamphetamine (Mohaghegh *et al.*, 1997). For example, Gordon *et al.* (1991) demonstrated that, while rectal temperature was still significantly higher in MDMA-treated animals 3 h post-injection (approximately 4 °C above control values), tail skin temperature was approximately 2 °C lower than in saline-treated rats. These authors also exposed a drug-naïve group to a high T_a environment (37 °C) for 60 min, and observed a significant increase in both rectal (+3 °C) and tail (+8 °C) temperature, compared to saline-treated rats. The authors concluded that MDMA treatment resulted in a failure to increase blood flow to the tail, a common thermoregulatory response to an increase in body temperature (see Grant, 1963) which was demonstrated in the group exposed to a high T_a (Gordon *et al.*, 1991). Although it is difficult to make a direct comparison between the current study and that

of Gordon *et al.* (1991), as the latter did not report the pattern of change in rectal and tail temperature but only the values measured 3 h post-injection, the results are nevertheless similar. Therefore, based on the results of the current study and those of Gordon *et al.* (1991), MDMA treatment appears to interfere with normal heat dissipation mechanisms.

It has previously been suggested (see Shankaran & Gudelsky, 1999) that the acute release of 5-HT which occurs following MDMA administration to rats (see Colado & Green, 1994; Mechan *et al.*, 2000; Sabol & Seiden, 1998; Schmidt *et al.*, 1987; Schmidt & Taylor, 1988; Stone *et al.*, 1986; 1987b) is involved in mediation of the acute hyperthermic response. Thus, a number of 5-HT receptor antagonists were administered in an attempt to identify the receptor subtype(s) involved. Administration of the non-selective 5-$HT_{1/2}$ receptor antagonist, methysergide (10 mg/kg), had no effect on the MDMA-induced acute hyperthermic response, neither did pretreatment with the 5-HT_2 antagonist ritanserin (1 mg/kg). The lack of effect of ritanserin is in contrast to the reported effects of ketanserin, a 5-HT_2 receptor antagonist with structural similarities. For example, (1) Nash *et al.* (1988) administered ketanserin (3 mg/kg i.p.) to male SD rats 1 h prior to MDMA (3 or 10 mg/kg i.p.), and demonstrated a complete blockade of the MDMA-induced acute hyperthermic response, and (2) Malberg *et al.* (1996) administered ketanserin (6 mg/kg i.p.) to male Holtzman rats 1 h prior to administration of MDMA (40 mg/kg s.c.), and observed a hypothermic response in the ketanserin + MDMA treatment group, while ketanserin alone did not alter rectal temperature. There is some question as to the selectivity of ketanserin, however, which has also been demonstrated to have an antagonistic action at central α_1-adrenoceptors (see Dudley *et al.*, 1988; McCall & Schuette, 1984).

Administration of another 5-HT_2 antagonist, MDL 11,939 (5 mg/kg), resulted in a complete blockade of the hyperthermic response of MDMA-treated animals, without affecting the rectal temperature of control animals. This result is in agreement with the data of Schmidt *et al.* (1990a), where the same dose of MDL 11,939 blocked or significantly attenuated hyperthermia induced by MDMA (10, 20 and 30 mg/kg) in SD rats, and prevented MDMA-induced regional brain depletions of 5-HT measured one

week later. However, in addition to its high selectivity and affinity for the 5-HT_2 receptor binding site, MDL 11,939 does have moderate affinity for the α-1 adrenergic receptor, although it is 35 and 80 times less potent than cyproheptadine and ketanserin, respectively (see Dudley *et al.*, 1988).

In the current study, pretreatment with the selective 5-HT_{2C} receptor antagonist, SB 242084 (3 mg/kg), had no effect on MDMA-induced acute hyperthermia. At the lower dose administered (0.1 mg/kg) the 5-HT_{2A} antagonist, MDL 100,907, also had no effect on the hyperthermic response. However, a modest attenuation was observed following the higher dose (0.3 mg/kg).

Administration of two serotonin reuptake inhibitors, zimeldine (10 mg/kg) and fluoxetine (10 mg/kg), had no effect on MDMA-induced acute hyperthermia. However, fluoxetine has been demonstrated to almost completely abolish the MDMA-induced release of 5-HT within the hippocampus, as measured via *in vivo* microdialysis (Mechan *et al.*, 2001b). The results of the current study are in agreement with Malberg *et al.* (1996), who demonstrated that fluoxetine prevented MDMA-induced regional brain depletion of 5-HT and 5-HIAA measured 14 days post-treatment, without altering the acute hyperthermic response. In addition, Berger *et al.* (1992) demonstrated that fluoxetine pretreatment significantly attenuated the synaptosomal release of [^{3}H]5-HT, as induced by MDMA, PCA, methamphetamine and fenfluramine. The current results are also in agreement with a study performed in humans using another serotonin uptake inhibitor, citalopram: Liechti & Vollenweider (2000) demonstrated that citalopram pretreatment (40 mg in 500 ml saline solution), 90 min prior to oral administration of MDMA (1.5 mg/kg), attenuated MDMA-induced increases in blood pressure and heart rate, while having no effect on MDMA-induced increases in body temperature.

In addition to 5-HT, dopamine is acutely released following MDMA administration to rats (see Colado *et al.*, 1999a; Crespi *et al.*, 1997; Gudelsky *et al.*, 1994; Koch & Galloway, 1997; Johnson *et al.*, 1986; Nash, 1990; Nash & Brodkin, 1991; Sabol & Seiden, 1998; Schmidt *et al.*, 1992; Shankaran & Gudelsky, 1999; Yamamoto & Spanos, 1988). Furthermore, it has been postulated that stimulation of 5-HT_2 receptors

following MDMA-induced 5-HT release might serve to enhance dopamine release, at least in the striatum (see Gudelsky *et al.*, 1994; Nash, 1990; Schmidt *et al.*, 1990b). Thus a series of compounds which affect dopaminergic function were administered in an attempt to determine whether dopamine receptors are involved in the mediation of the MDMA-induced hyperthermic response in rats.

Pretreatment with the dopamine D_2 receptor antagonist, remoxipride (10 mg/kg), did not affect the MDMA-induced acute hyperthermia. However, the D_1 antagonist, SCH 23390 (0.3, 1 and 2 mg/kg), dose-dependently attenuated the hyperthermic response. In fact, at the highest dose, hypothermia occurred in the SCH 23390 + MDMA treatment group, while the temperature of control animals was unaltered. These results are consistent with the observations of Metzger *et al.* (2000), who reported that SCH 23390 pretreatment attenuated methamphetamine-induced hyperthermia in SD rats. In addition, Bronstein & Hong (1995) demonstrated a delayed hypothermic response in SD rats administered SCH 23390 (0.5 mg/kg s.c.) and methamphetamine (10 mg/kg s.c.); although the peak hyperthermic response was not attenuated, hypothermia was observed in the SCH 23390 + methamphetamine treatment group 5 h post-injection. The authors suggested that the protective effects of SCH 23390 pretreatment against methamphetamine-induced lethality could be due to this delayed hypothermic action (Bronstein & Hong, 1995). There is also some evidence for an interaction of SCH 23390 with central 5-HT_2 receptors: Bischoff *et al.* (1986) reported partial inhibition of *in vivo* [^{3}H]spiperone binding in the rat frontal cortex. However, such an effect was not observed in the striatum or hippocampus, and 45 - 50 % inhibition in the frontal cortex was only observed following administration of 3 and 10 mg/kg SCH 23390 - no significant effect was seen at the doses used in the current study (Bischoff *et al.*, 1986).

There is additional support for the hypothermic effects of SCH 23390 in previous work demonstrating the involvement of D_2 receptors in temperature responses in rats. For example, Faunt & Crocker (1987) showed that administration of the primarily D_2-selective agonist, apomorphine (0.25 - 1 μMol/kg), resulted in a dose-dependent hypothermia. Following administration of apomorphine (1 μMol/kg), a maximal decrease in temperature of ˜1.2 °C was observed 30 min post-injection, an effect which

was potentiated by pretreatment with SCH 23390 (0.35 and 1.74 μMol/kg) (Faunt & Crocker, 1987). Thus, if hypothermia can be induced through dopamine D_2 receptor stimulation, it could be postulated that the hypothermic response observed in the MDMA + SCH 22390 treatment group, following the highest dose of SCH 23390 (2 mg/kg) in the current study, is due to complete blockade of D_1 receptors by the antagonist, enabling expression of D_2 receptor stimulation by MDMA-released dopamine.

Pretreatment with the dopamine uptake inhibitor, GBR 12909 (10 mg/kg) did not modify the MDMA-induced acute hyperthermic response, and has also been shown not to alter the MDMA-induced increase in extracellular dopamine, or decrease in extracellular HVA, in the striatum (Mechan *et al.*, 2001b). These results are not consistent with the reports of Koch & Galloway (1997), who demonstrated that GBR 12909 (1 μMol) completely blocked *in vitro* MDMA-induced dopamine release in rat striatal slices, and had no effect on MDMA-induced 5-HT release, or Nash & Brodkin (1991), who demonstrated that striatal dopamine release following central administration of MDMA (10 μMol) was significantly inhibited by systemic administration of the dopamine uptake inhibitors GBR 12909 (10 mg/kg i.p.) and mazindol (5 mg/kg i.p.). In addition, Marek *et al.* (1990) demonstrated that methamphetamine-induced neostriatal dopamine release was prevented by pretreatment with a high dose of mazindol (40 mg/kg i.p.), while a 20 mg/kg dose had no effect. However, differences in methodology might explain the different results obtained in the current study, compared to those previously reported: in particular, comparisons between *in vivo* and *in vitro* results (Koch & Galloway, 1997), and the fact that both MDMA and GBR 12909 were administered peripherally in the current study, in contrast to central administration of MDMA by Nash & Brodkin (1991). The current results are consistent with those observed in mice, where GBR 12909 also failed to protect against acute MDMA-induced dopamine depletion (O'Shea *et al.*, 2001). While it could be argued that the dose of GBR 12909 used in the current study (10 mg/kg) was too low to alter MDMA-induced effects, nevertheless it is comparable to doses used in some previous studies (e.g. Nash & Brodkin, 1991; Rothman *et al.*, 1991).

The majority of work described in this chapter strongly indicated the lack of direct involvement of 5-HT receptors in the MDMA-induced acute hyperthermic response - a non-selective 5-$HT_{1/2}$ antagonist (methysergide), a 5-HT_2 antagonist (ritanserin) and two serotonin uptake inhibitors (zimeldine and fluoxetine) had no effect on this response. However, a second 5-HT_2 antagonist (MDL 11,939) and a selective 5-HT_{2A} antagonist (MDL 100, 907) did modify the response, thus indicating some involvement of the 5-HT_{2A} receptor. Although MDL 100,907 has been reported to have a lack of affinity for dopamine D_2 receptors (see Kehne *et al.*, 1996a; 1996b), it has been demonstrated to attenuate dopamine synthesis and release. For example, Schmidt *et al.* (1992) reported that pretreatment with MDL 100,907 (0.01 - 1 mg/kg s.c.) 30 min prior to administration of MDMA (20 mg/kg s.c) to SD rats, completely prevented MDMA-induced stimulation of striatal dopamine synthesis, and striatal dopamine release was significantly attenuated. In addition, Pehek *et al.* (2001) demonstrated that MDL 100,907 (10 and 100 μMol) significantly attenuated *in vivo* potassium-stimulated dopamine release within the medial prefrontal cortex (mPFC) of SD rats.

Therefore, it could be postulated that the hyperthermic response which results from MDMA administration is mediated through a combination of 5-HT_{2A} and dopamine D_1 receptors: (1) 5-HT released by MDMA acts directly at 5-HT_{2A} receptors, in addition to indirectly enhancing the actions of MDMA-released dopamine at D_1 receptors, (2) MDL 100,907 directly antagonises serotonergic stimulation of 5-HT_{2A} receptors, and attenuates MDMA-induced dopamine release, and (3) SCH 23390 directly antagonises dopaminergic stimulation of D_1 receptors. In combination, these postulated events could explain modification of the acute hyperthermia by both 5-HT_2/5-HT_{2A} receptor antagonists and a D_1 antagonist. This hypothesis does not, however, explain the lack of effect of another 5-HT_2 antagonist (ritanserin) and a 5-$HT_{1/2}$ antagonist (methysergide) - perhaps MDL 11,939 has additional actions at receptors other than the 5-HT_2 receptor (such as the α-1 receptor) and perhaps the modification of the hyperthermic response seen at the higher dose of MDL 100,907 was due to its attenuation of dopamine release.

In conclusion, the current study demonstrated that the acute hyperthermia which follows MDMA administration to rats is not solely mediated by 5-HT receptors, but that

dopamine D_1 receptors play a major role in the hyperthermic response. Recreational MDMA use by humans can be followed by an extreme hyperthermia (see Chadwick *et al.*, 1991; Henry *et al.*, 1992; Mallick & Bodenham, 1997; Randall, 1992; Screaton *et al.*, 1992), which might lead to disseminated intravascular coagulation and rhabdomyolysis (see Chadwick *et al.*, 1991; Henry *et al.*, 1992; Screaton *et al.*, 1992). Current treatment tends to involve cooling the body with ice (see Brown & Osterloh, 1987; Mallick & Bodenham, 1997; Screaton *et al.*, 1992), or administration of the peripherally-acting muscle relaxant dantrolene (see Mallick & Bodenham, 1997; Tehan, 1993), which might act by reducing thermogenesis (see Barrett, 1992). Thus it is plausible that a dopamine D_1 antagonist could be administered as a more specific preventative measure against the life-threatening consequences of the hyperthermic response.

CHAPTER 7

NEUROPROTECTION:

A STUDY OF THE MECHANISMS INVOLVED IN MDMA-INDUCED STRIATAL DOPAMINE LOSS IN NIH/S MICE

7 NEUROPROTECTION: A study of the mechanisms involved in MDMA-induced striatal dopamine loss in NIH/S mice

7.1 INTRODUCTION

The acute and longer-term neurochemical changes which occur following MDMA administration to rats and mice have been described in Chapter 1. The current chapter is concerned with the mechanisms involved in the longer-term neurotoxic action of MDMA on dopaminergic systems in the mouse brain. Therefore, literature concerning the longer-term effects of MDMA administration to mice, and comparison to the effects seen in rats, will be highlighted here. In addition, the use of drugs with putative neuroprotective abilities and the neuroprotective effects of preventing MDMA-induced hyperthermia, as reported in rats and mice, will be reviewed.

7.1.1 Longer-term neurotoxic effects of MDMA administration: comparisons between mice and rats

Administration of MDMA to rats results in long-term neurotoxic damage to regional brain serotonergic systems, as evidenced by selective degeneration of forebrain axon terminals and decreases in 5-HT and 5-HIAA content, tryptophan hydroxylase activity and [^{3}H]paroxetine binding (see Battaglia *et al.*, 1987; 1988; 1991; Colado *et al.*, 1995; 1997a; 1999a; Commins *et al.*, 1987; O'Hearn *et al.*, 1988; Schmidt, 1987a; Schmidt *et al.*, 1987; Stone *et al.*, 1986). However, long-term neurotoxic changes in brain dopaminergic systems have been reported not to occur following MDMA administration to rats (Battaglia *et al.*, 1987; 1991; Colado *et al.*, 1997a; Schmidt *et al.*, 1987; Stone *et al.*, 1986). In contrast, MDMA selectively damages dopaminergic systems in mice, while having little effect on brain 5-HT content (Laverty & Logan, 1990; Logan *et al.*, 1988; Miller & O'Callaghan, 1993; 1994; 1995; O'Callaghan & Miller, 1994).

Stone *et al.* (1987a) demonstrated potential differences in MDMA pharmacokinetics between species, longer-term deficits in serotonergic activity only being seen in mice

where multiple doses of MDMA were administered, compared to rats where a single dose had a long-lasting effect. For example, administration of MDMA (10 mg/kg s.c.) to rats resulted in decreases in tryptophan hydroxylase activity and 5-HT and 5-HIAA concentrations, which were still 20 - 40 % below control values one week later. In mice, an acute decrease in 5-HT and 5-HIAA concentrations was observed 3 h post-treatment, but values returned to control levels within 24 h. However, administration of multiple doses of MDMA to mice (15 mg/kg s.c., six times, at 4 h intervals) resulted in reductions in hippocampal tryptophan hydroxylase activity and 5-HT and 5-HIAA concentrations, which were still 25 - 30 % below control values one week later (Stone *et al.*, 1987a).

Logan *et al.* (1988) demonstrated marked differences in the longer-term neurotoxic effects of MDMA between rats and mice. Administration of multiple doses of MDMA (25 mg/kg i.p., three times, at 12 h intervals) to rats resulted in an approximately 50 % decrease in cortical 5-HT and 5-HIAA, which was still apparent seven days post-treatment, while there was no effect on striatal dopamine and DOPAC concentrations. In mice, however, only small decreases were seen in cortical 5-HIAA and striatal DOPAC concentrations. Administration of a higher dose of MDMA to mice (50 mg/kg i.p., three times, at 12 h intervals) resulted in a prolonged decrease in cortical 5-HIAA concentration, levels being approximately 80 % of control values two weeks post-treatment, while both dopamine and DOPAC concentrations were diminished by approximately 50 %, an effect which persisted for at least two weeks following administration of MDMA (Logan *et al.*, 1988).

Laverty & Logan (1990) extended their earlier work (Logan *et al.*, 1988) by investigating the effects of several pretreatment compounds on MDMA-induced neurotoxicity in rats and mice. MK-801 (1 mg/kg i.p.) was administered 30 min before MDMA (50 mg/kg i.p.) for a total of three times in 24 h. Seven days later the neurotoxic effects of MDMA, as indicated by reductions in striatal and cortical concentrations of 5-HT and 5-HIAA in rats and by reductions in striatal dopamine and its metabolites in mice, were shown to be significantly attenuated in MK-801-pretreated animals. Furthermore, in mice, pretreatment with haloperidol (5 mg/kg i.p.) or α-

methyl-p-tyrosine (200 mg/kg ester i.p.) also prevented the MDMA-induced reduction in striatal dopamine, DOPAC and HVA concentrations, whereas pretreatment with fluoxetine (10 mg/kg i.p.) had no effect (Laverty & Logan, 1990).

Miller & O'Callaghan (1993) investigated the biochemical effects of MDMA, in addition to methamphetamine and fenfluramine, in female C57BL/6 mice, by measuring the levels of glial fibrillary acidic protein (GFAP) in the cortex, hippocampus and striatum. Central nervous system injury results in astrocytic hypertrophy and, therefore, enhanced expression of the astrocyte-localised GFAP (see Miller & O'Callaghan, 1993). MDMA (25 mg/kg s.c.) was administered a total of four times, at 2 h intervals, and regional levels of GFAP were shown to be significantly increased 48 h later. The authors also investigated the involvement of glutamate in MDMA-induced neurotoxicity, by pretreating animals with the NMDA antagonist, MK-801 (1 mg/kg s.c., 30 min prior to the first and third doses of MDMA). The increases in striatal GFAP were completely prevented by MK-801 in MDMA-treated animals, thus indicating a potential role for excitatory amino acids in the MDMA-induced neurotoxic injury (Miller & O'Callaghan, 1993).

O'Callaghan & Miller (1994) further extended their earlier work (Miller & O'Callaghan, 1993) by investigating additional measures of neurotoxicity. Female C57BL/6J mice were administered multiple doses of MDMA (20 mg/kg s.c., four times, at 2 h intervals) which resulted in: (1) significant increases in GFAP concentration in the striatum and, to a lesser extent, in the cortex, (2) rapid decreases in striatal tyrosine hydroxylase and dopamine concentrations and smaller decrements in cortical 5-HT concentration, and (3) argyrophilic axons and fine debris in the striatum, particularly the rostral, dorsal and lateral aspects. These results indicated that MDMA administration resulted in striatal and cortical damage and, in particular, that dopaminergic projections to the striatum were the primary targets of degeneration (O'Callaghan & Miller, 1994). In addition to the protective effects of MK-801 pretreatment against the MDMA-induced increase in striatal GFAP (Miller & O'Callaghan, 1993; O'Callaghan & Miller, 1994), the decreases in striatal tyrosine hydroxylase and dopamine concentrations, as measured three days post-treatment, were also prevented by MK-801 (O'Callaghan &

Miller, 1994). However, it was later shown that the neuroprotective action of MK-801 was likely to be due to its ability to reduce core temperature (Miller & O'Callaghan, 1994).

Miller & O'Callaghan (1994) investigated the effects of altering core temperature on the neurotoxic effects of MDMA, in addition to methamphetamine and MDA. Female C57BL/6J mice were administered MDMA (20 mg/kg s.c.) a total of four times, at 2 h intervals, and biochemical measurements were made three days later. MDMA-induced acute hyperthermia, decrease in striatal dopamine concentration and increase in GFAP were all prevented both by lowering ambient temperature (from 22 °C to 15 °C) and by pretreatment with MK-801 (Miller & O'Callaghan, 1994; 1995). The hypothermic and neuroprotective effects of MK-801 were also observed both in male C57BL/6J mice and in female B6C3F1 mice, indicating that the effects seen were not gender- or strain-specific (Miller & O'Callaghan, 1995). By increasing ambient temperature to 27 °C, the effects of lowering ambient temperature and MK-801 were completely reversed and significantly attenuated, respectively. Additional compounds reported to cause hypothermia (ethanol, pentobarbital, diethyldithiocarbamate (DDC), fenfluramine or cocaine) were administered prior to MDMA, to investigate whether their temperature-altering effects also affected MDMA-induced neurotoxicity. In general, compounds which induced greater reductions in core temperature provided greater neuroprotection. For example, ethanol and pentobarbital, which had similar hypothermic effects to MK-801, significantly blocked the MDMA-induced decrease in striatal dopamine concentration and increase in GFAP. Thus it was clearly demonstrated that, by preventing MDMA-induced hyperthermia, neurotoxicity was also prevented or significantly attenuated (Miller & O'Callaghan, 1994).

O'Shea *et al.* (2001) investigated the effects of pretreatment with GBR 12909 and fluoxetine on the neurochemical effects of MDMA administration to male Swiss Webster mice, in an attempt to assess the involvement of 5-HT and dopamine transporters in MDMA-induced neurotoxicity. MDMA (10, 20 or 30 mg/kg i.p.) was administered for a total of three doses at 3 h intervals and, seven days later, significant reductions in striatal dopamine, DOPAC and HVA were observed in animals

administered the two higher doses of MDMA. In contrast, no effects on regional brain concentrations of 5-HT or 5-HIAA were observed. Pretreatment with GBR 12909 (10 mg/kg i.p. 30 min before each 30 mg/kg MDMA injection) prevented the MDMA-induced reduction in striatal dopamine, DOPAC and HVA concentrations, measured seven days post-treatment. However, pretreatment with fluoxetine (10 mg/kg i.p. 30 min before each MDMA injection) did not significantly attenuate the MDMA-induced reduction in striatal concentrations of dopamine and its metabolites. While the selective dopamine uptake inhibitor GBR 12909 protected against longer-term striatal dopamine loss, it had no effect on MDMA-induced acute hyperthermia. Thus the neuroprotective effect of GBR 12909 did not result from any effect on temperature (O'Shea *et al.*, 2001).

A number of studies have investigated the effects of restraint on MDMA-induced hyperthermia and reductions in striatal dopamine concentration, restraint being employed to characterise the physiological consequences of "stress" (see Johnson *et al.*, 2000; Miller & O'Callaghan, 1994; 1995). For example, Johnson *et al.* (2000) administered MDMA (20 mg/kg s.c., four times, at 2 h intervals) to female C57BL/6 mice 30 min after restraint commenced. The restraint procedure comprised placing each mouse in a 35 ml centrifuge tube (25 mm diameter, 89 mm length), with perforations in the closed end to provide airflow and a clip secured across the open end to prevent the mouse backing out of the tube. The apparatus enabled movement from supine to prone position. Rectal temperature was measured prior to the animal being placed in the restraint apparatus, each time the animal was removed from the apparatus for an injection, and at the end of the experiment (2 h after the last injection). H.p.l.c. analysis of dopamine, 5-HT, noradrenaline and their metabolites was performed three days later. Restraint prevented the MDMA-induced acute hyperthermic response, inducing hypothermia in both MDMA- and saline-treated mice. MDMA-treated, unrestrained mice demonstrated an 82 % reduction in striatal dopamine, DOPAC and HVA. Restraint substantially blocked MDMA-induced reductions in striatal dopamine, while having no effect on dopamine levels in saline-treated mice; MDMA-treated, restrained mice demonstrated a 32 % decrement in striatal dopamine concentration. Thus, the induction of "stress" in mice, in producing hypothermia, served to protect against the

neurotoxic effects of MDMA shown by preventing MDMA-induced reductions in striatal dopamine concentration (Johnson *et al.*, 2000; Miller & O'Callaghan, 1994; 1995) and increases in striatal GFAP (Miller & O'Callaghan, 1994; 1995).

7.1.2 Aims of the investigation: the use of putative neuroprotective compounds

The current study was undertaken in an attempt to elucidate the mechanisms involved in the neurotoxic action of MDMA in mice, there being few reports on the effects of MDMA in mice, in contrast to the substantial number of studies on rats. Following the study of O'Shea *et al.* (2001) where the selective dopamine uptake inhibitor GBR 12909 was demonstrated to have neuroprotective effects on longer-term MDMA-induced reductions in striatal dopamine, a range of putative neuroprotective agents were chosen to investigate different aspects of the neurotoxic pathway. In addition, as temperature has been demonstrated to play an important role in the neuroprotective abilities of certain compounds (e.g. Ali *et al.*, 1994; Buchan & Pulsinelli, 1990; Callahan & Ricaurte, 1998; Colado *et al.*, 1993; 1995; 1998; 1999b; 1999c; Farfel & Seiden, 1995a; 1995b; Hewitt & Green, 1994; Kita *et al.*, 2000; Miller & O'Callaghan, 1994; 1995; Murray *et al.*, 1996), rectal temperature was monitored regularly throughout each experiment.

Three or four doses of MDMA (see Colado *et al.*, 1999c; Laverty & Logan, 1990; Logan *et al.*, 1988; Miller & O'Callaghan, 1993; 1994; 1995; O'Callaghan & Miller, 1994; O'Shea *et al.*, 2001; Stone *et al.*, 1987a) or methamphetamine (see Miller & O'Callaghan, 1993; Sonsalla *et al.*, 1989; 1991) have generally been reported as necessary to produce neurotoxic damage in mice. Therefore, a preliminary study was conducted to confirm the necessity for multiple doses to induce dopaminergic neurotoxicity, whereby mice were administered one, two or three doses of MDMA and striatal levels of dopamine and its metabolites were analysed one week later. All other experiments involved the administration of three doses of MDMA (25 mg/kg i.p.) at 3 h intervals.

The compounds studied were:

(1) The low affinity non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist AR-R15896AR, which has previously been shown to be neuroprotective in animal models of focal and global ischaemia (Cregan *et al.*, 1997), but did not protect against MDMA-induced neurodegenerative loss of 5-HT or 5-HIAA in the hippocampus, striatum or cortex of the rat (Colado *et al.*, 1998).

(2) The non-competitive NMDA antagonist MK-801, which is neuroprotective in animal models of ischaemia (e.g. Buchan & Pulsinelli, 1990) and against MDMA- (Colado *et al.*, 1993; Colado & Green, 1994; Farfel & Seiden, 1995a; Hewitt & Green, 1994), methamphetamine- (Baldwin *et al.*, 1993; Bowyer *et al.*, 1994; Farfel & Seiden, 1995b; Green *et al.*, 1992; Johnson *et al.*, 1989) and *p*-chloroamphetamine- (Farfel & Seiden, 1995b) induced neurotoxicity in rats and MDMA-, MDA- (Miller & O'Callaghan, 1993; 1995; O'Callaghan & Miller, 1994) and methamphetamine- (Albers & Sonsalla, 1995; Ali *et al.*, 1994; Boireau *et al.*, 1995; Miller & O'Callaghan, 1993; 1995; O'Callaghan & Miller, 1994, Sonsalla *et al.*, 1989; 1991) induced neurotoxicity in mice. However, MK-801 has been demonstrated to induce hypothermia, and therefore its neuroprotective effects have been attributed to its ability to lower core temperature (Albers & Sonsalla, 1995; Ali *et al.*, 1994; Bowyer *et al.*, 1994; Buchan & Pulsinelli, 1990; Farfel & Seiden, 1995a; 1995b; Miller & O'Callaghan, 1994; 1995).

(3) The GABAmimetic clomethiazole, which is neuroprotective in animal models of ischaemia (Baldwin *et al.*, 1994; Cross *et al.*, 1991; Green, 1998) and against MDMA- (Colado *et al.*, 1993; 1998; 1999b; Colado & Green, 1994; Hewitt & Green, 1994) and methamphetamine- (Baldwin *et al.*, 1993; Green *et al.*, 1992) induced neurotoxicity in rats and methamphetamine-induced neurotoxicity in mice (Green *et al.*, 1992). Although clomethiazole has been demonstrated to attenuate the MDMA-induced hyperthermic response (Colado *et al.*, 1993; 1998; 1999b; Hewitt & Green, 1994), it appears that some additional neuroprotective mechanism is involved. Colado *et al.* (1998) maintained the rectal temperature of rats pretreated with clomethiazole near that of MDMA-treated animals and demonstrated that, although the degree of protection was diminished, it was still present.

(4) The novel, and relatively selective, nitric oxide synthase (NOS) inhibitor AR-R17477AR, which has been shown to be protective in animal models of global and focal ischaemia (O'Neill *et al.*, 2000).

(5) The NOS inhibitor S-methyl-L-thiocitrulline (S-MTC), which has been demonstrated to protect against methamphetamine-induced dopaminergic neurotoxicity in mice (Itzhak *et al.*, 2000).

(6) The NOS inhibitor 7-nitroindazole (7-NI), which protects against methamphetamine- (Ali & Itzhak, 1998; Callahan & Ricaurte, 1998; Di Monte *et al.*, 1996; Itzhak & Ali, 1996; Itzhak *et al.*, 2000) and MDMA- (Colado *et al.*, 1999c) induced neurotoxicity in mice. However, some studies have shown that the neuroprotective effects of 7-NI are likely to be due to its ability to prevent methamphetamine- (Callahan & Ricaurte, 1998) or MDMA- (Colado *et al.*, 1999c) induced hyperthermia.

(7) The spin trap reagent α-phenyl-*N*-tert-butyl nitrone (PBN), which significantly attenuates MDMA- (Colado & Green, 1995; Colado *et al.*, 1997a; Yeh *et al.*, 1999) and PCA- (Murray *et al.*, 1996) induced neurotoxicity in rats and MPTP- (Ferger *et al.*, 2000) and methamphetamine- (Kita *et al.*, 2000) induced neurotoxicity in mice. However, some studies have shown that PBN prevents MDMA- (Colado & Green, 1995), PCA- (Murray *et al.*, 1996) and methamphetamine- (Kita *et al.*, 2000) induced hyperthermia, while others have shown modest neuroprotection without altering MDMA-induced acute hyperthermia (Colado *et al.*, 1997a).

7.2 METHODS

7.2.1 Animals, drug administration and temperature measurement

Adult male NIH/S mice were used for all experiments, except in the 7-NI treatment experiment where Swiss Webster mice were employed, and were housed as detailed in sections 2.1(1) and 2.1(3).

Pretreatment compounds were administered 30 min prior to each dose of MDMA (25 mg/kg i.p.) or saline. This regimen was repeated for a total of three times, at 3 h

intervals, the same dose of pretreatment compound being employed on each occasion, unless otherwise stated. In the S-MTC and 7-NI experiments, a lower dose of MDMA (20 mg/kg i.p.) was used, as the higher dose caused some mortality. However, reductions in striatal dopamine and hyperthermic responses were of similar magnitude to those seen in all other experiments, indicating that the main effects of MDMA were similar to those seen in other parts of the study. Drugs were administered i.p. unless otherwise specified. All drugs were administered in normal saline unless otherwise stated and doses are quoted as the base weight.

Rectal temperature was measured, as detailed in section 2.7, at regular intervals during all experiments. For the experiment where the effect of different doses of MDMA was examined, an M5003 digital thermometer was employed which resulted in the recording of lower actual temperature values. However, the changes in rectal temperature were similar to those seen in all other experiments.

7.2.2 Effect of different doses of MDMA on striatal monoamine concentrations

In order to assess the effect of different total doses of MDMA on striatal monoamine concentration, the protocol employed for all other experiments was modified. In this experiment, groups of mice (n = 6 in each case) were administered either MDMA (25 mg/kg i.p.) or saline three times at 3 h intervals (0 h, 3 h, 6 h), as follows: (1) MDMA was administered at each time-point; (2) MDMA was administered at 0 h and 3 h and saline was injected at 6 h; (3) MDMA was administered at 0 h and saline was injected at 3 h and 6 h; (4) saline was administered at each time-point.

7.2.3 Measurement of monoamines and their metabolites in cerebral tissue

One week following treatment, mice were sacrificed and regional brain tissue was processed as described in section 2.8. 5-HT, dopamine and their metabolites were subsequently measured by h.p.l.c. with electrochemical detection, as described in section 2.9.6.

7.2.4 Statistics

Striatal concentrations of dopamine and its metabolites were analysed using one-way ANOVA, followed by the Tukey multiple comparison post-hoc test, and regional brain concentrations of 5-HT and 5-HIAA were analysed using an unpaired *t*-test (GraphPad Prism). Statistical analyses of temperature data involved ANOVA with repeated measures (programme 2V) or, where missing values occurred, an unbalanced repeated measures model (programme 5V) was used. TREATMENT was the between subjects factor and TIME was the repeated measure. ANOVA was performed on both pre-treatment and post-treatment data (BMDP/386 Dynamic).

7.3 RESULTS

7.3.1 Effect of different doses of MDMA on striatal monoamine concentrations and rectal temperature

Mice administered a single dose of MDMA (25 mg/kg i.p. at 0 h) demonstrated a mean 3 % decline in striatal dopamine levels, which was not statistically significant. Administration of two doses of MDMA (25 mg/kg i.p. at 0 and 3 h) resulted in a 57 % decrease in striatal dopamine concentration compared to control animals, while mice administered three doses of MDMA (25 mg/kg i.p. at 0, 3 and 6 h) demonstrated a 71 % decrease in striatal dopamine concentration compared to saline-treated control mice (Figures 7.1a and 7.1c). There was no statistically significant difference in the loss of striatal dopamine between mice administered two or three doses of MDMA. Similar reductions were seen in the striatal concentrations of DOPAC and HVA (Figure 7.1b), while no changes in striatal concentrations of 5-HT or 5-HIAA were observed following any of the doses of MDMA (Table 7.1).

A single dose of MDMA produced a rise in rectal temperature which lasted for over 3 h, while temperature had returned to control values 4 h after administration (Figure 7.2a). Mice administered two doses of MDMA (at 0 h and 3 h) demonstrated a similar rise in temperature to the first group, this rise being sustained until after the second dose and then declined after 5 h, reaching control values 6.5 h after the first dose (Figure 7.2b). Mice administered three doses of MDMA demonstrated a rise in temperature following

the first dose which was further increased after the second dose. Rectal temperature declined towards control values prior to the third dose, after which a rapid increase was again observed (Figure 7.2c).

7.3.2 Effect of MDMA on regional brain 5-HT and 5-HIAA concentrations

Administration of three doses of MDMA did not significantly alter concentrations of 5-HT or 5-HIAA in the striatum, hypothalamus, hippocampus or cortex as measured seven days post-treatment, in any of the experiments performed (for example see Table 7.2). In addition, this lack of effect was not altered by any of the pretreatment compounds investigated (for example see Figure 7.3).

7.3.3 Effect of NMDA antagonists on MDMA-induced striatal dopamine loss and hyperthermia

The low affinity non-competitive NMDA antagonist AR-R15896AR (20 mg/kg i.p. before the first MDMA dose and 5 mg/kg i.p. before the second and third doses) did not protect against MDMA-induced striatal dopamine loss, there being no difference in dopamine concentration between MDMA and AR-R15896AR + MDMA treatment groups (Figure 7.4a). MDMA-induced reductions in striatal concentrations of DOPAC and HVA were also not modified by AR-R15896AR pretreatment (Table 7.3). AR-R15896AR had no effect on the acute hyperthermic response following the first two injections of MDMA but abolished the rise seen following the third dose (Figure 7.4b).

The high affinity non-competitive NMDA antagonist, MK-801 (0.5 mg/kg i.p.) also afforded no protection against MDMA-induced reductions in striatal dopamine concentration (Figure 7.5a). This compound had no overall statistically significant effect on MDMA-induced hyperthermia, although the MDMA-induced temperature rise following the third dose was prevented by MK-801 pretreatment (Figure 7.5b). There were no statistically significant differences between MDMA- and saline-treatment groups with respect to striatal concentrations of DOPAC or HVA (Table 7.4).

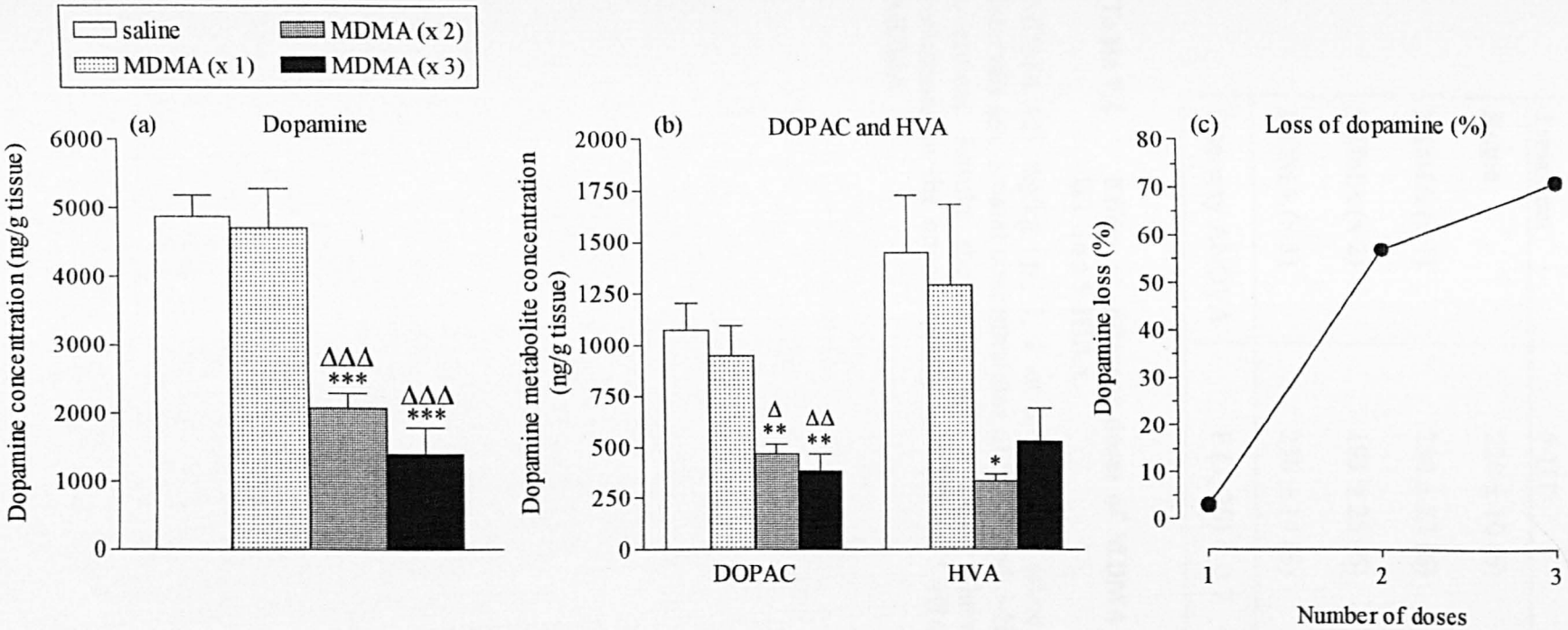

Figure 7.1 Effect of different doses of MDMA on striatal concentrations of dopamine and its metabolites.

MDMA (25 mg/kg i.p., 1, 2 or 3 doses) or saline was administered 3 times at 3 h intervals and striatal concentrations of dopamine, DOPAC and HVA were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 5 - 6 in each group. ***(a) Dopamine.*** There was a significant difference between treatment groups ($F(3, 20) = 21.2$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels in MDMA (x 2) and MDMA (x 3) treatment groups compared to saline-treated control mice (***$p < 0.001$) and compared to the MDMA (x 1) treatment group ($^{\Delta\Delta\Delta}p < 0.001$). ***(b) DOPAC and HVA.*** There was a significant difference between treatment groups in the concentrations of DOPAC ($F(3, 20) = 10.0$, $p < 0.001$) and HVA ($F(3, 20) = 4.8$, $p < 0.05$). Tukey's multiple comparison test demonstrated a significant reduction in: (1) DOPAC levels in MDMA (x 2) and MDMA (x 3) treatment groups compared to saline-treated control mice (**$p < 0.01$) and compared to the MDMA (x 1) treatment group ($^{\Delta}p < 0.05$; $^{\Delta\Delta}p < 0.01$), and (2) HVA levels in the MDMA (x 2) treatment group compared to saline-treated control mice (*$p < 0.05$). ***(c) Loss of striatal dopamine following 1, 2 or 3 doses of MDMA, calculated as a % of control values.***

Treatment	Indole concentration (ng/g tissue) 5-HT	5-HIAA
Saline	220 ± 10 (6)	208 ± 21 (6)
MDMA (x 1)	230 ± 23 (6)	219 ± 34 (5)
MDMA (x 2)	193 ± 25 (6)	199 ± 41 (6)
MDMA (x 3)	210 ± 14 (5)	241 ± 13 (5)
One-way ANOVA	F (3, 20) = 0.7	F (3, 20) = 0.4

Table 7.1 Effect of different doses of MDMA on striatal concentrations of 5-HT and 5-HIAA.

MDMA (25 mg/kg i.p., 1, 2 or 3 doses) or saline was administered 3 times at 3 h intervals and striatal concentrations of 5-HT and 5-HIAA were measured 1 week post-treatment. Results shown as mean ± s.e.m. There were no statistically significant reductions in the concentrations of 5-HT or 5-HIAA following any of the doses of MDMA.

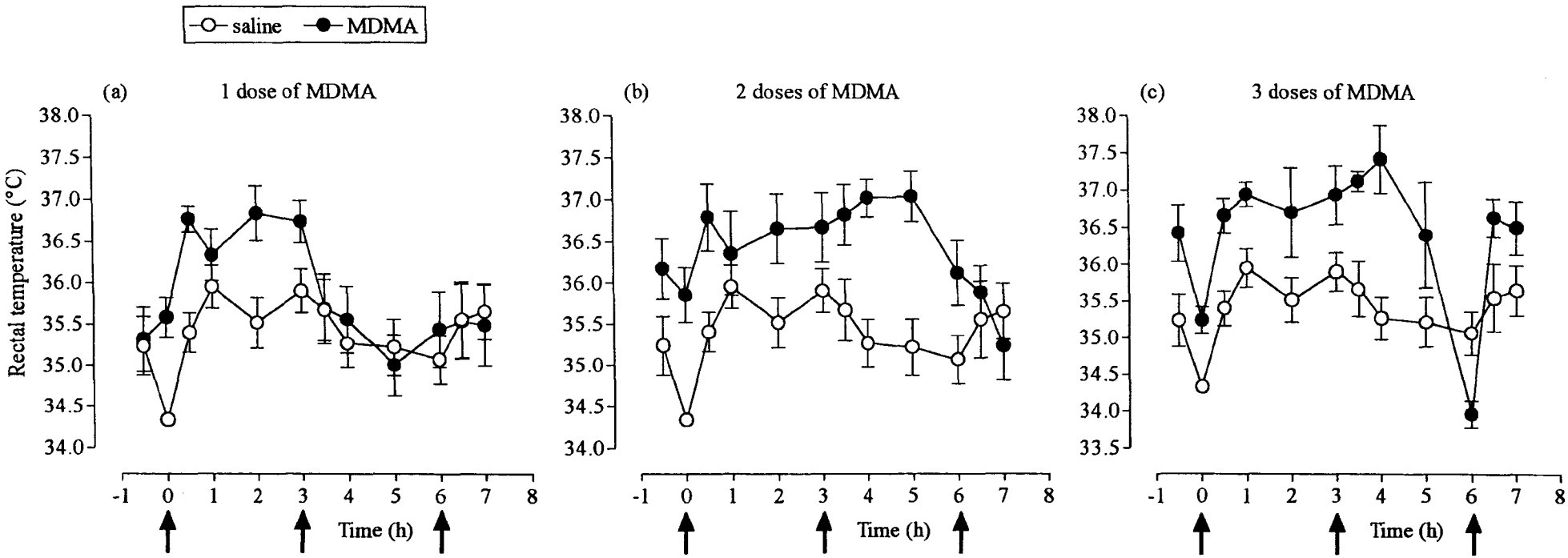

Figure 7.2 Effect of different doses of MDMA on rectal temperature.

MDMA (25 mg/kg i.p., 1, 2 or 3 doses) or saline was administered 3 times at 3 h intervals (denoted by arrows). Results shown as mean ± s.e.m., n = 5 - 6 in each group. ***(a) Effect of 1 dose of MDMA.*** MDMA was administered at 0 h and saline was administered at 3 and 6 h. MDMA-treated animals demonstrated a significant rise in rectal temperature compared to control mice, during $t_{0-3.5}$ ($F(1, 10) = 12.2$, $p < 0.01$). ***(b) Effect of 2 doses of MDMA.*** MDMA was administered at 0 and 3 h and saline was administered at 6 h. MDMA-treated animals demonstrated a significant rise in rectal temperature compared to control mice ($F(1, 10) = 14.4$, $p < 0.01$). ***(c) Effect of 3 doses of MDMA.*** MDMA was administered at all 3 time-points (0, 3 and 6 h). MDMA-treated animals demonstrated a significant rise in rectal temperature compared to control mice ($F(1, 9) = 32.5$, $p < 0.01$).

	Indole concentration (ng/g tissue)					
	Indole	Saline	MDMA	% loss	t	p
Striatum	5-HT	314 ± 18	275 ± 34	12	1.1	0.3
	5-HIAA	257 ± 24	267 ± 21	~	0.3	0.8
Hypothalamus	5-HT	494 ± 52	422 ± 56	15	1.0	0.4
	5-HIAA	387 ± 56	345 ± 60	11	0.5	0.6
Hippocampus	5-HT	357 ± 40	344 ± 19	4	0.3	0.8
	5-HIAA	312 ± 22	345 ± 23	~	1.0	0.3
Cortex	5-HT	425 ± 56	350 ± 18	18	1.2	0.3
	5-HIAA	165 ± 22	144 ± 8	13	0.8	0.4

Table 7.2 Effect of MDMA on regional brain concentrations of 5-HT and 5-HIAA.

Mice were administered MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and regional brain concentrations of 5-HT and 5-HIAA were measured 1 week post-treatment. Results shown as mean ± s.e.m. There were no statistically significant reductions in the concentrations of 5-HT or 5-HIAA in any of the brain regions examined.

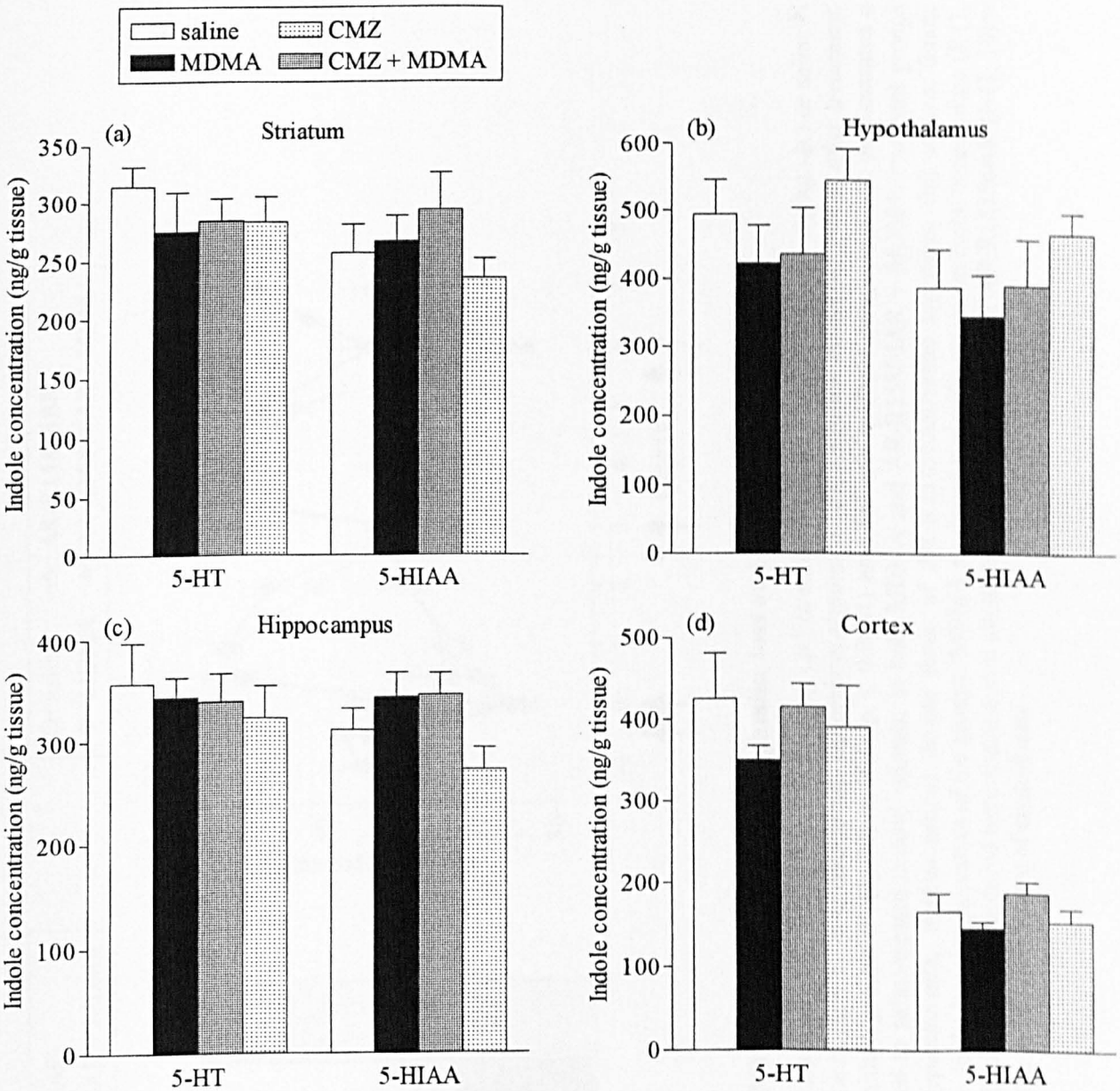

Figure 7.3 Effect of clomethiazole (CMZ) pretreatment on regional brain 5-HT and 5-HIAA concentrations following MDMA administration.

Mice were administered CMZ (50 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and regional brain concentrations of 5-HT and 5-HIAA were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There were no differences between treatment groups with respect to concentrations of 5-HT or 5-HIAA in all brain regions examined (striatum, hypothalamus, hippocampus, cortex).

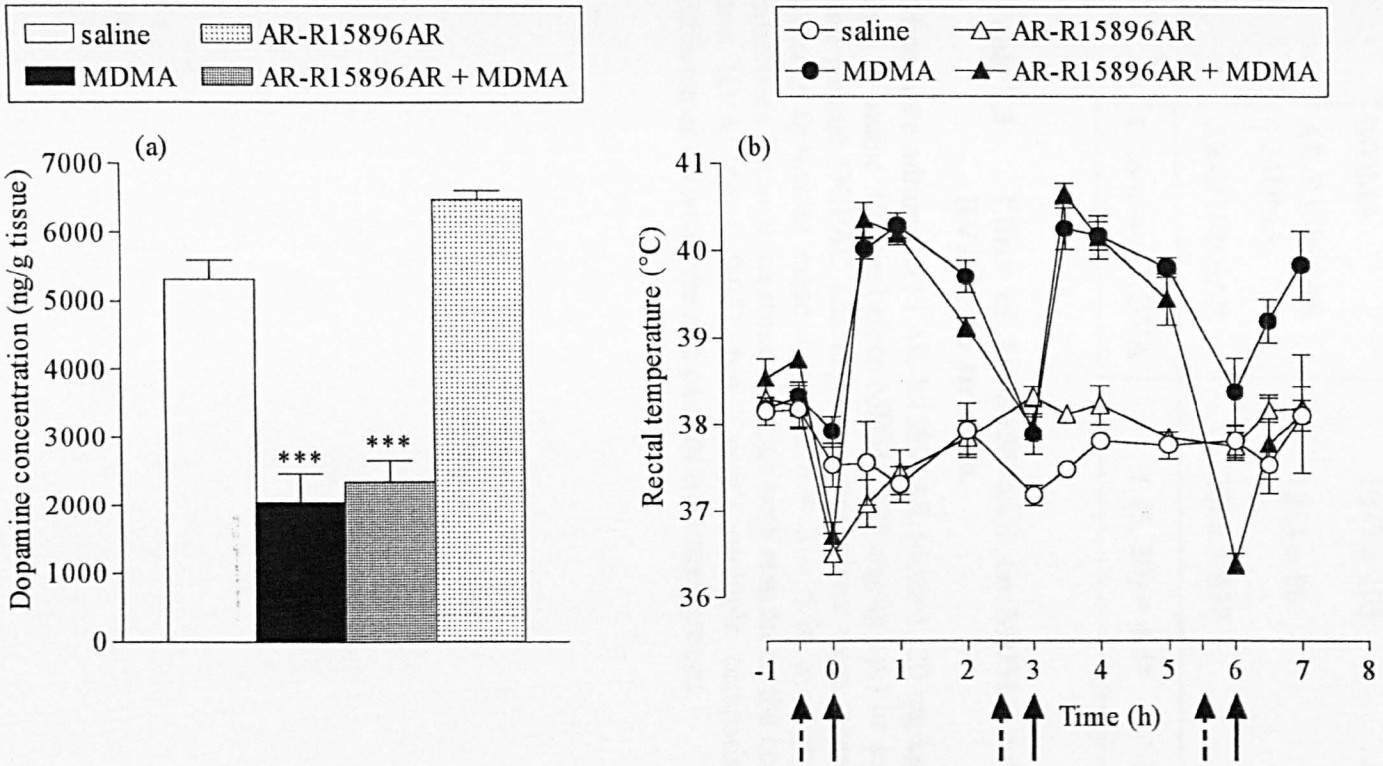

Figure 7.4 Effect of AR-R15896AR on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered AR-R15896AR (dose 1, 20 mg/kg i.p.; doses 2 and 3, 5 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 20) = 58.6$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels compared to saline-treated control animals, in both MDMA and AR-R15896AR + MDMA treatment groups (***$p < 0.001$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of AR-R15896AR/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 8) = 196.8$, $p < 0.001$) compared to saline-treated mice. MDMA-induced hyperthermia was attenuated after the third dose of AR-R15896AR ($F(1, 10) = 18.2$, $p < 0.01$). AR-R15896AR had no effect on the rectal temperature of control mice.

Treatment	Dopamine metabolite concentration (ng/g tissue) DOPAC	HVA
Saline	2364 ± 538	931 ± 125
MDMA	1077 ± 195	645 ± 103
AR-R15896AR + MDMA	1248 ± 88	754 ± 42
AR-R15896AR	2588 ± 638	1049 ± 141
One-way ANOVA	F (3, 20) = 4.5*	F (3, 20) = 3.5*

Table 7.3 Effect of AR-R15896AR on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered AR-R15896AR (dose 1, 20 mg/kg i.p.; doses 2 and 3, 5 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There was a significant difference between treatment groups with respect to the concentrations of both DOPAC and HVA (*$p < 0.05$), but Tukey's multiple comparison test did not reveal any differences between specific pairs of treatment groups.

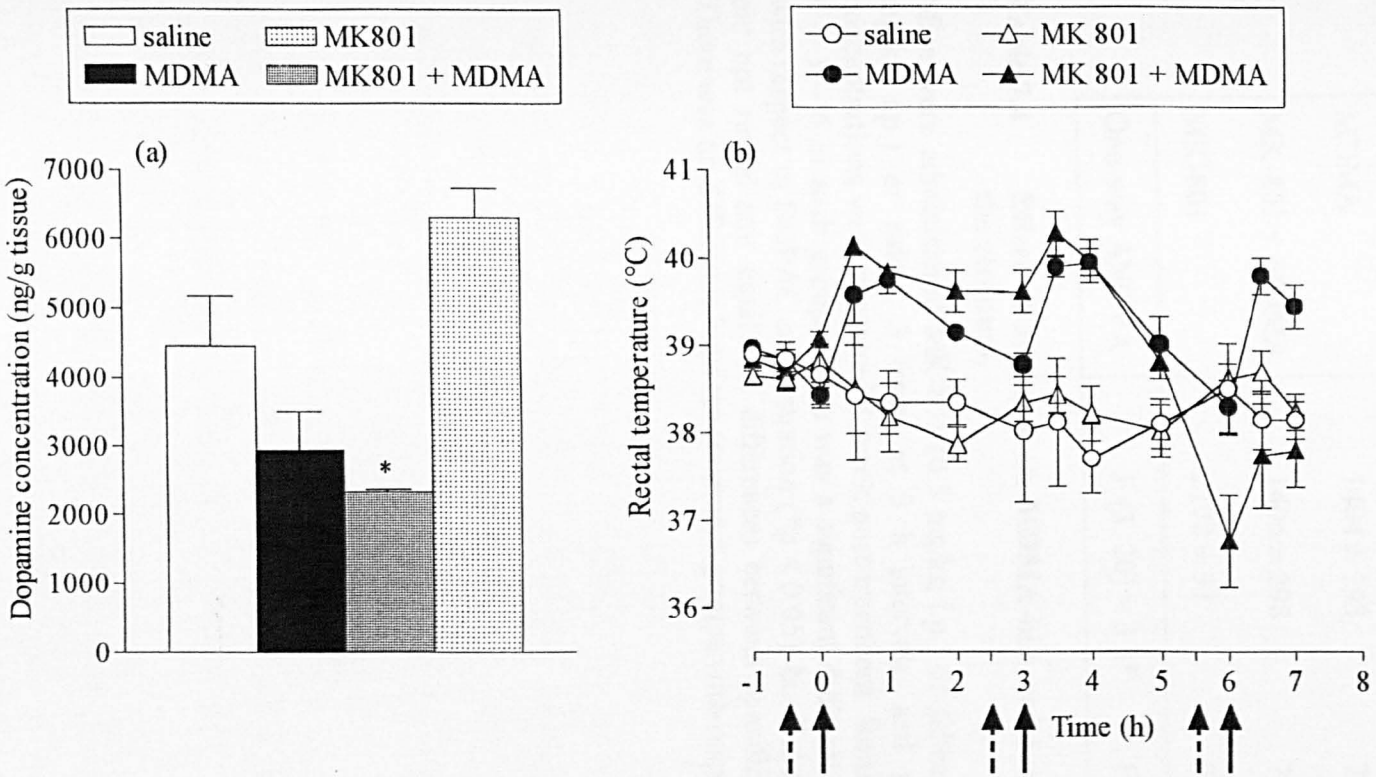

Figure 7.5 Effect of MK-801 on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered MK-801 (0.5 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 20) = 14.9$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels compared to saline-treated control animals, in the MK-801 + MDMA treatment group (*$p < 0.05$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of MK-801/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 8) = 8.8$, $p < 0.05$) compared to saline-treated mice. MK-801 did not modify MDMA-induced hyperthermia and had no effect on the rectal temperature of control mice.

Treatment	Dopamine metabolite concentration (ng/g tissue) DOPAC	HVA
Saline	1923 ± 235	884 ± 101
MDMA	1404 ± 193	755 ± 92
MK-801 + MDMA	1406 ± 298	757 ± 111
MK-801	2172 ± 91	977 ± 67
One-way ANOVA	F (3, 20) = 3.8*	F (3, 20) = 1.6

Table 7.4 Effect of MK-801 on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered MK-801 (0.5 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There was a significant difference between treatment groups with respect to DOPAC concentration (*$p < 0.05$), but Tukey's multiple comparison test did not reveal any significant differences between specific pairs of treatment groups. There was no difference between treatment groups with respect to HVA concentration.

7.3.4 Effect of clomethiazole on MDMA-induced striatal dopamine loss and hyperthermia

The GABAmimetic compound clomethiazole (CMZ; 50 mg/kg i.p.) did not protect against MDMA-induced striatal dopamine loss (Figure 7.6a) and there were no statistically significant differences between treatment groups with respect to striatal levels of DOPAC or HVA (Table 7.5). Following each dose of clomethiazole, a hypothermic response was observed in both MDMA- and saline-treated mice (Figure 7.6b). However, apart from after the third dose of clomethiazole where hypothermia was sustained for over 1 h, CMZ + MDMA-treated mice still demonstrated peak hyperthermic responses similar to those of MDMA-treated mice (Figure 7.6b).

7.3.5 Effect of NOS inhibitors on MDMA-induced striatal dopamine loss and hyperthermia

The novel, and relatively selective, nNOS inhibitor AR-R17477AR (5 mg/kg s.c.) provided complete protection against MDMA-induced striatal dopamine loss (Figure 7.7a). There were no statistically significant differences between treatment groups with respect to DOPAC or HVA concentrations, although both metabolites were reduced in MDMA-treated mice (Table 7.6). Following each dose of AR-R17477AR, a hypothermic response was observed in MDMA- treated mice (Figure 7.7b). However, apart from after the third dose of AR-R17477AR, AR-R17477AR + MDMA-treated mice still demonstrated peak hyperthermic responses which corresponded to the temperature response of MDMA-treated mice (Figure 7.7b).

The NOS inhibitor S-methyl-L-thiocitrulline (S-MTC; 10 mg/kg i.p.) also provided significant neuroprotection against striatal dopamine loss (Figure 7.8a), without altering the MDMA-induced hyperthermic response (Figure 7.8b).

Pretreatment with the NOS inhibitor 7-nitroindazole (7-NI; 50 mg/kg i.p.) produced substantial protection against MDMA-induced striatal dopamine loss (Figure 7.9a). However, in contrast to the other two NOS inhibitors used, 7-NI also induced a marked hypothermic response in both MDMA- and saline-treated animals (Figure 7.9b).

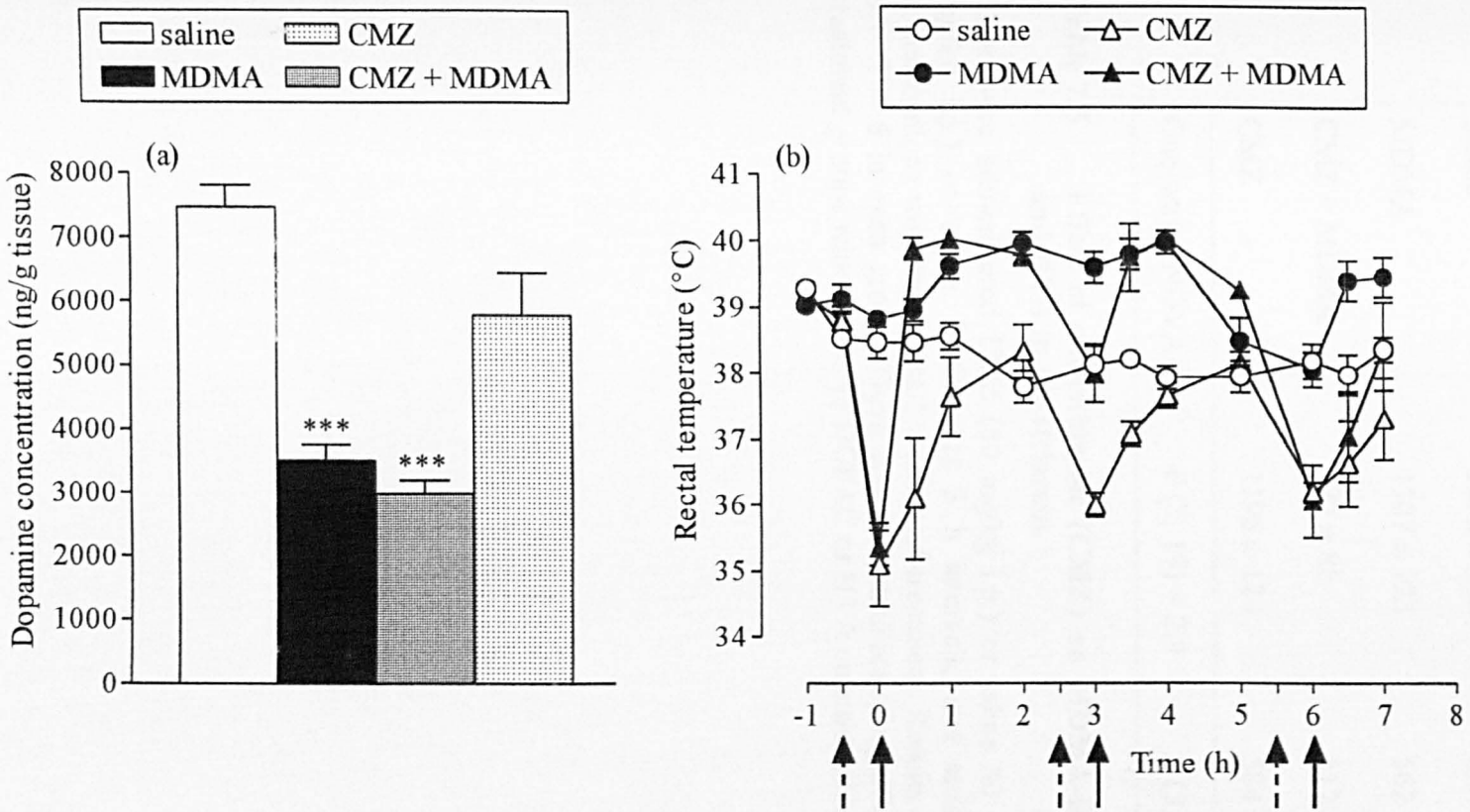

Figure 7.6 Effect of clomethiazole (CMZ) on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered CMZ (50 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 20) = 26.1$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels compared to saline-treated control mice, in both MDMA and CMZ + MDMA treatment groups (***$p < 0.001$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of CMZ/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 9) = 20.9$, $p < 0.01$) compared to saline-treated mice. CMZ induced a hypothermic response in both MDMA-treated ($F(1, 9) = 7.5$, $p < 0.05$) and saline-treated mice ($F(1, 10) = 12.7$, $p < 0.01$).

Treatment	Dopamine metabolite concentration (ng/g tissue) DOPAC	HVA
Saline	1230 ± 155	654 ± 67
MDMA	1107 ± 123	562 ± 51
CMZ + MDMA	777 ± 85	512 ± 50
CMZ	1196 ± 124	584 ± 39
One-way ANOVA	F (3, 19) = 2.9	F (3, 19) = 1.3

Table 7.5 Effect of clomethiazole (CMZ) on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered CMZ (50 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There were no statistically significant differences between treatment groups with respect to DOPAC or HVA concentrations.

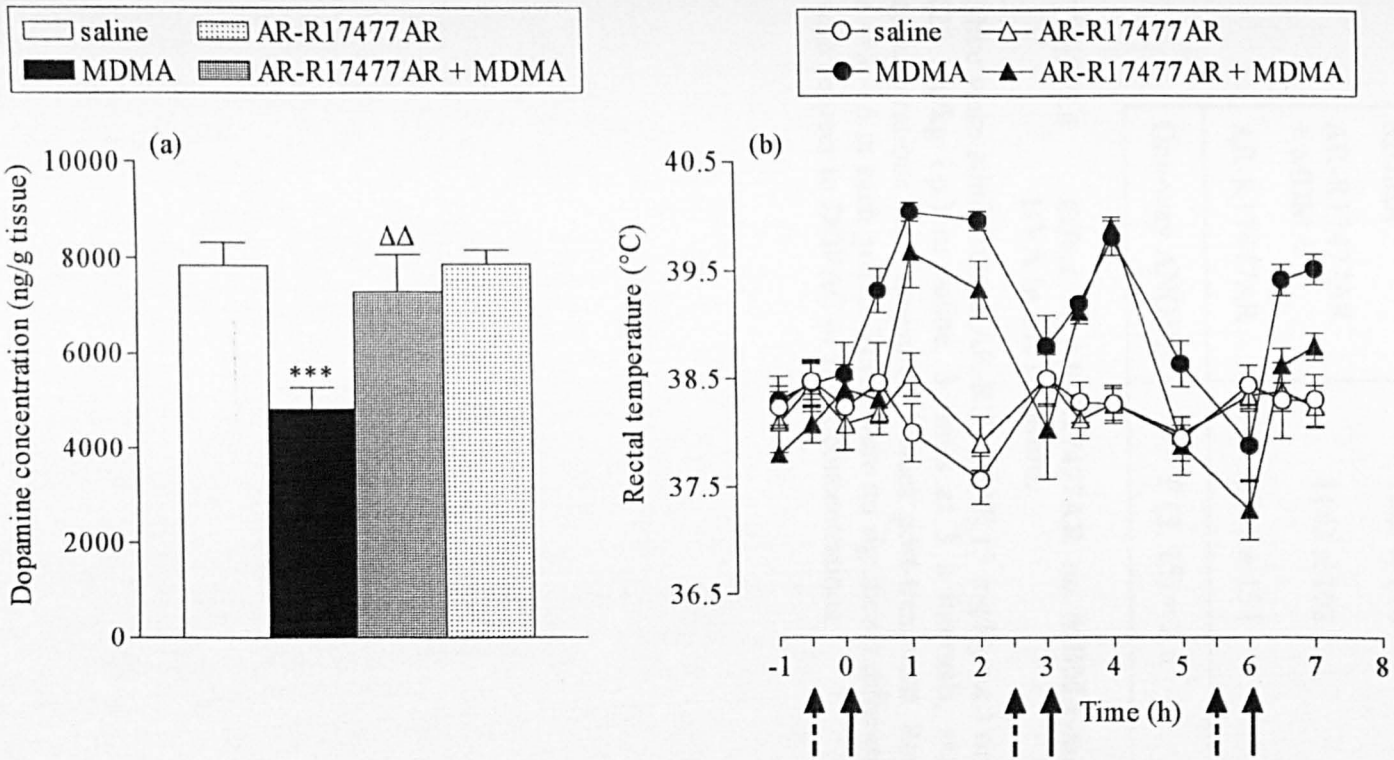

Figure 7.7 Effect of AR-R17477AR on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered AR-R17477AR (5 mg/kg s.c.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 20) = 9.7$, $p < 0.001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels compared to saline-treated control mice, in MDMA-treated animals (***$p < 0.001$). AR-R17477AR protected against this reduction (AR-R17477AR + MDMA different from MDMA, $^{\Delta\Delta}p < 0.01$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of AR-R17477AR/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 8) = 18.0$, $p < 0.01$) compared to saline-treated mice. AR-R17477AR induced a hypothermic response in MDMA-treated mice, after the third dose ($F(1, 7) = 68.6$, $p < 0.001$).

Treatment	Dopamine metabolite concentration (ng/g tissue)	
	DOPAC	HVA
Saline	1406 ± 81	914 ± 40
MDMA	1021 ± 116	731 ± 59
AR-R17477AR + MDMA	1143 ± 108	855 ± 76
AR-R17477AR	1241 ± 151	903 ± 59
One-way ANOVA	F (3, 15) = 2.0	F (3, 15) = 2.2

Table 7.6 Effect of AR-R17477AR on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered AR-R17477AR (5 mg/kg s.c.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There were no significant differences between treatment groups with respect to DOPAC or HVA concentrations.

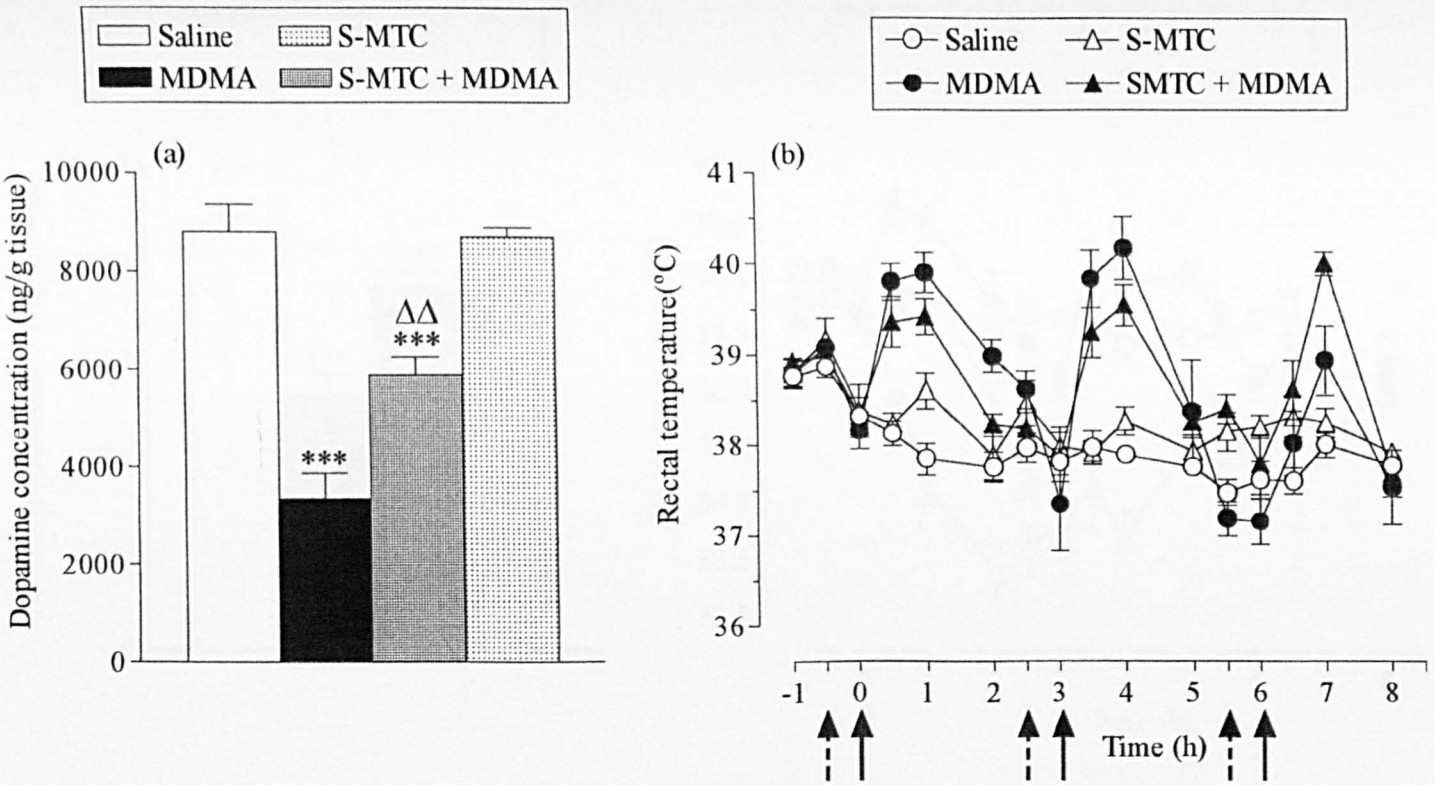

Figure 7.8 Effect of S-methyl-L-thiocitrulline (S-MTC) on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered S-MTC (10 mg/kg i.p.) or saline 30 min before MDMA (20 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 6 - 10 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 24) = 9.7$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels compared to saline-treated control mice, in both MDMA and S-MTC + MDMA treatment groups (***$p < 0.001$). S-MTC provided significant protection against this reduction (S-MTC + MDMA different from MDMA, $^{\Delta\Delta}p < 0.01$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of S-MTC/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 16) = 17.2$, $p < 0.001$) compared to saline-treated mice. S-MTC did not modify the MDMA-induce hyperthermic response and had no effect on the rectal temperature of control mice.

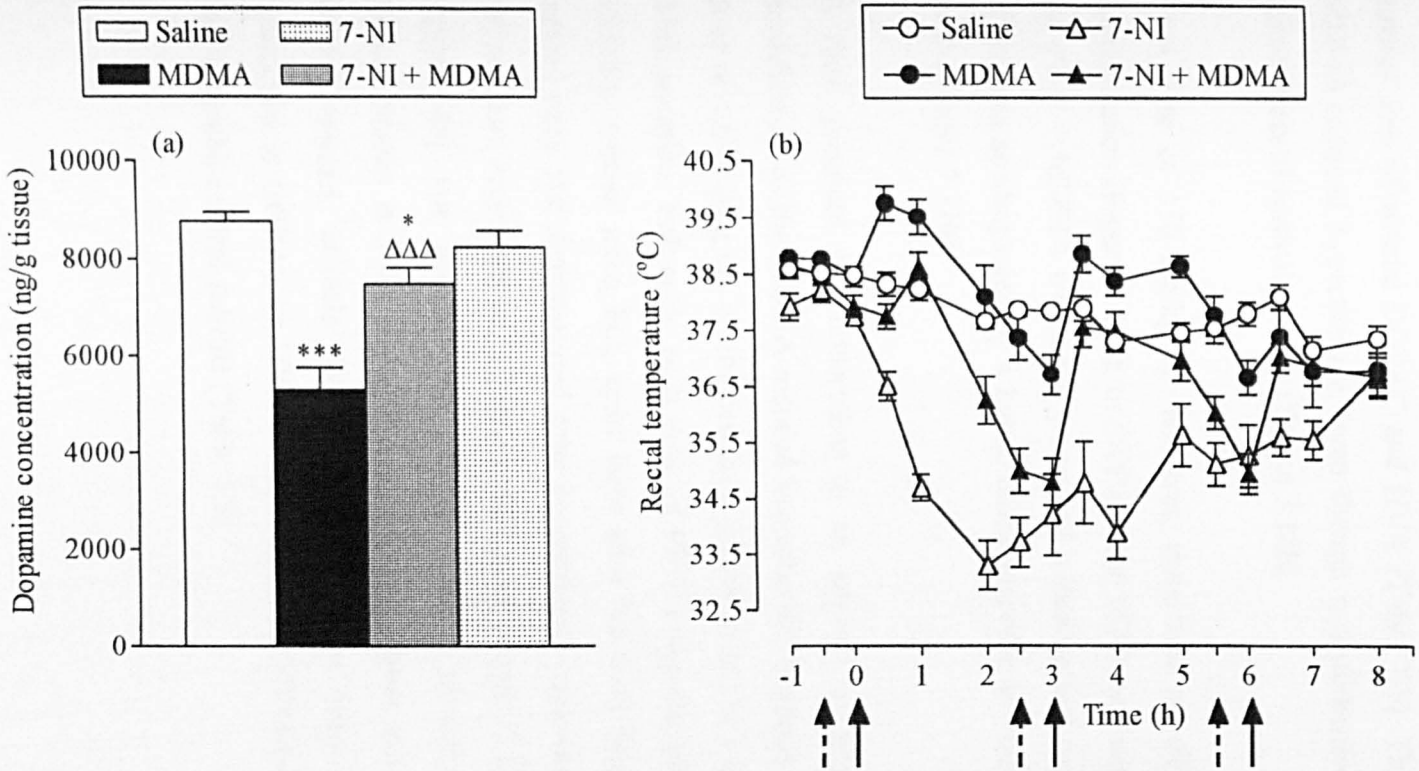

Figure 7.9 Effect of 7-nitroindazole (7-NI) on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered 7-NI (50 mg/kg i.p.) or saline 30 min before MDMA (20 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 6 - 10 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 40) = 17.9$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels compared to saline-treated control mice, in both MDMA (***$p < 0.001$) and 7-NI + MDMA (*$p < 0.05$) treatment groups. 7-NI provided significant protection against this reduction (7-NI + MDMA different from MDMA, $^{\Delta\Delta\Delta}p < 0.001$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of 7-NI/saline injections, full arrows denote MDMA/saline injections. MDMA produced a significant rise in rectal temperature ($F(1, 22) = 7.6$, $p < 0.01$) compared to saline-treated mice. 7-NI abolished the MDMA-induced hyperthermic response ($F(1, 25) = 24.3$, $p < 0.001$) and induced a frank hypothermia in saline-treated mice ($F(1, 16) = 169.0$, $p < 0.001$).

7.3.6 Effect of PBN on MDMA-induced striatal dopamine loss and hyperthermia

The nitrone free radical trapping agent α-phenyl-*N*-tert-butyl nitrone (PBN; 120 mg/kg i.p.) did not protect against MDMA-induced striatal dopamine loss (Figure 7.10a) or against loss of striatal DOPAC and HVA (Table 7.7). This dose also did not modify MDMA-induced hyperthermia, even though a hypothermic response was observed in saline-injected control animals (Figure 7.10b).

At a dose of 150 mg/kg i.p. however, there was no difference in striatal dopamine concentration (Figure 7.11a), or DOPAC and HVA concentrations (Table 7.8), between the PBN + MDMA treatment group and saline-treated control animals. However, this effect was accompanied by a hypothermic response in both MDMA- and saline-treated mice (Figure 7.11b).

A third protocol was undertaken in an attempt to attain neuroprotection without modification of the MDMA-induced hyperthermic response. PBN was administered at a dose of 120 mg/kg i.p. on the first two occasions and at a dose of 150 mg/kg i.p. on the third occasion. Following each dose of PBN a hypothermic response was observed in MDMA- treated mice, but, apart from after the third dose of PBN, PBN + MDMA-treated mice still demonstrated peak hyperthermic responses which corresponded to the temperature responses of MDMA-treated mice (Figure 7.12b). However, this treatment regime did not prevent the MDMA-induced striatal dopamine loss; dopamine concentrations in PBN + MDMA-treated mice were the same as those recorded in MDMA-treated animals (Figure 7.12a). There were no statistically significant reductions in DOPAC or HVA concentrations in MDMA-treated animals compared to saline-treated control animals (Table 7.9).

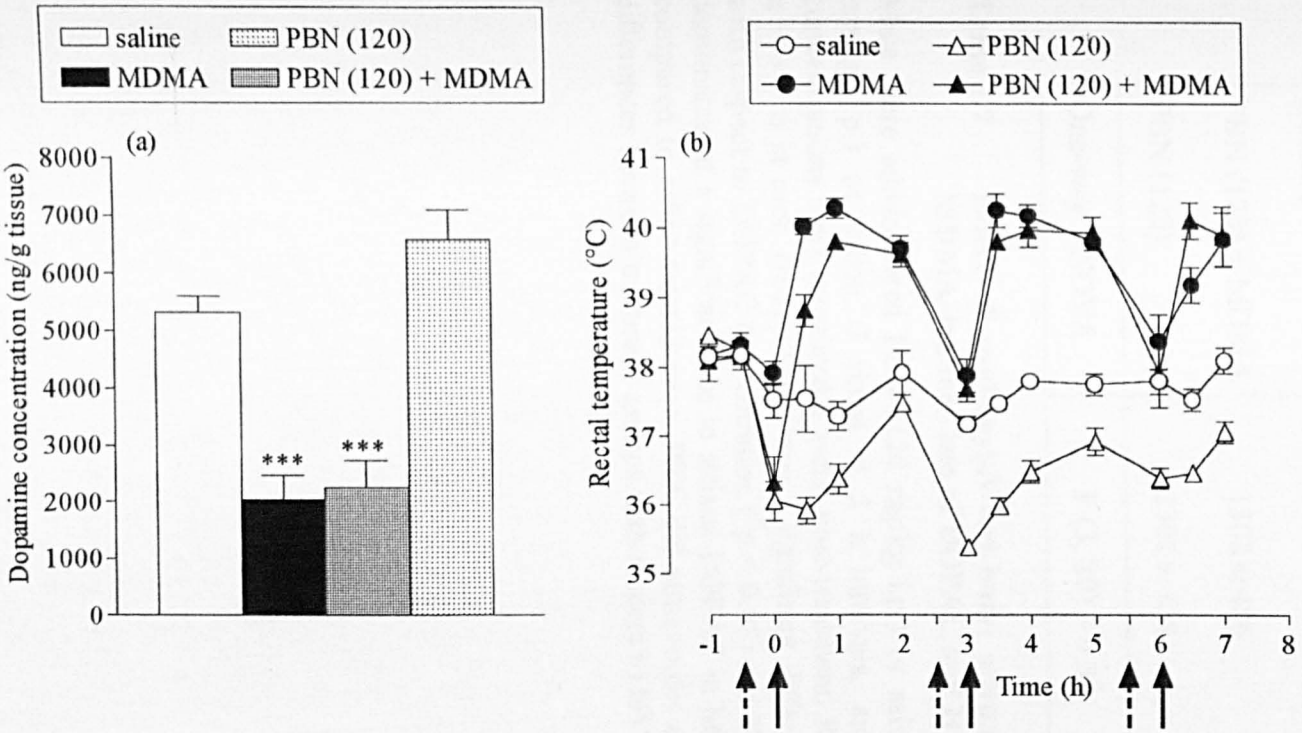

Figure 7.10 Effect of α-phenyl-*N*-tert-butyl nitrone (PBN, 120 mg/kg x 3) on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered PBN (120 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 20) = 33.5$, $p < 0.0001$) and Tukey's multiple comparison test demonstrated a significant reduction in dopamine levels in MDMA-treated animals, compared to saline-treated control mice, in both MDMA and PBN + MDMA treatment groups (***$p < 0.001$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of PBN/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 8) = 208.4$, $p < 0.001$) compared to saline-treated mice. PBN induced a hypothermic response in MDMA-treated mice, following the first dose ($F(1, 9) = 15.3$, $p < 0.01$) and in saline-treated mice after each dose ($F(1, 7) = 77.4$, $p < 0.001$).

Treatment	Dopamine metabolite concentration (ng/g tissue) DOPAC	HVA
Saline	2364 ± 538	930 ± 125
MDMA	1077 ± 195*	645 ± 103
PBN (120) + MDMA	1302 ± 486	657 ± 187
PBN (120)	2301 ± 338	1004 ± 95
One-way ANOVA	$F(3, 20) = 4.3^{\Delta}$	$F(3, 20) = 2.9$

Table 7.7 Effect of *α*-phenyl-*N*-tert-butyl nitrone (PBN, 120 mg/kg x 3) on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered PBN (120 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There was a significant difference between treatment groups with respect to DOPAC concentration ($^{\Delta}p < 0.05$) and Tukey's multiple comparison test demonstrated a significant loss in striatal DOPAC in MDMA-treated mice ($*p < 0.05$) compared to saline-treated mice. PBN did not protect against this loss. There were no differences between treatment groups with respect to HVA concentration.

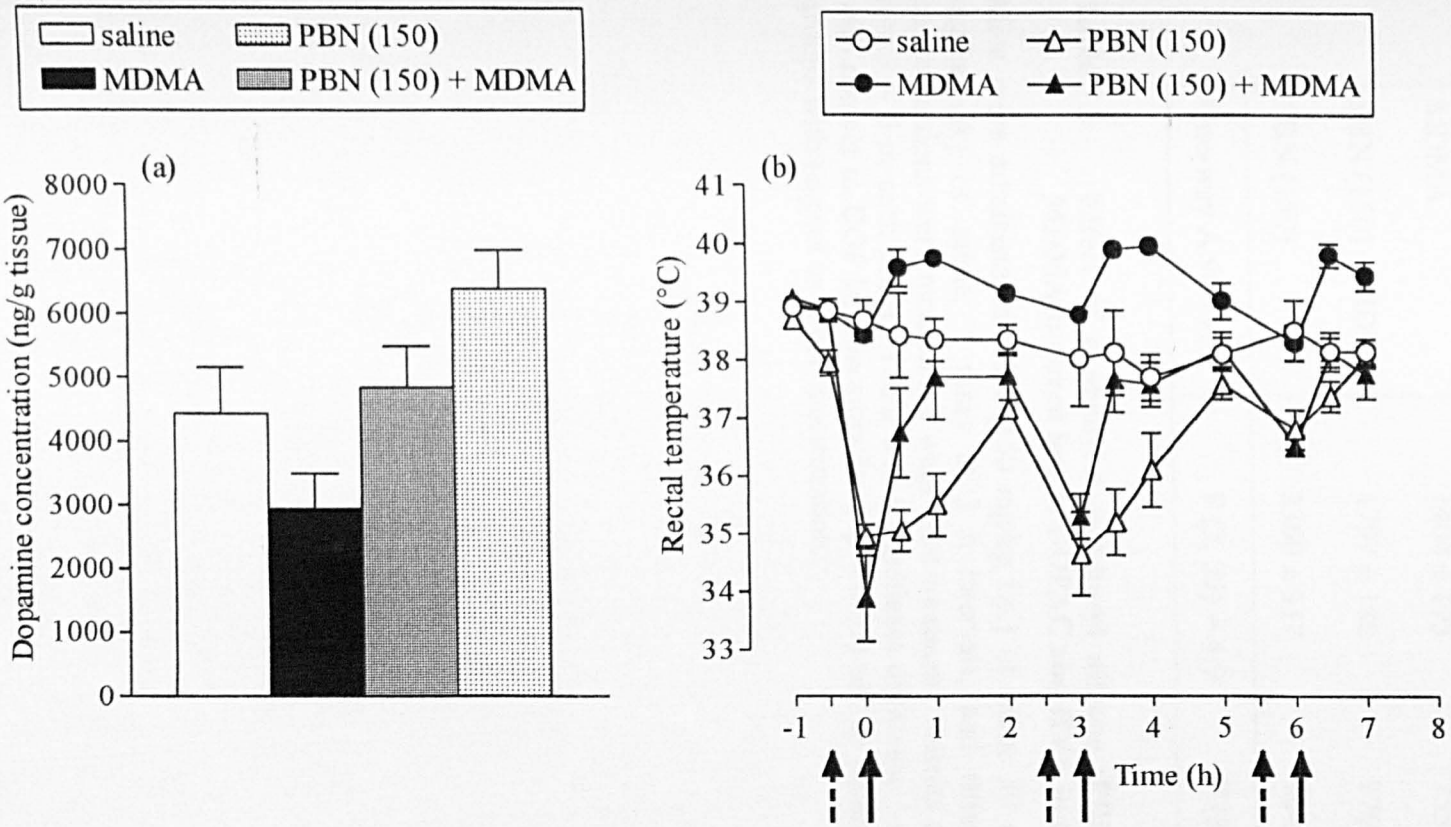

Figure 7.11 Effect of α-phenyl-*N*-tert-butyl nitrone (PBN, 150 mg/kg x 3) on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered PBN (150 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There was a significant difference between treatment groups ($F(3, 20) = 6.4$, $p < 0.01$). ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of PBN/saline injections, full arrows denote MDMA/saline injections. MDMA produced a significant rise in rectal temperature ($F(1, 8) = 8.8$, $p < 0.05$) compared to saline-treated mice. PBN induced a hypothermic response in both MDMA-treated ($F(1, 9) = 34.2$, $p < 0.001$) and saline-treated mice ($F(1, 6) = 14.7$, $p < 0.01$).

Treatment	Dopamine metabolite concentration (ng/g tissue) DOPAC	HVA
Saline	1923 ± 235	884 ± 101
MDMA	1404 ± 193	755 ± 92
PBN (150) + MDMA	1797 ± 188	976 ± 91
PBN (150)	2360 ± 317	1045 ± 63
One-way ANOVA	$F(3, 20) = 4.0^*$	$F(3, 20) = 2.6$

Table 7.8 Effect of α-phenyl-*N*-tert-butyl nitrone (PBN, 150 mg/kg x 3) on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered PBN (150 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There was a significant difference between treatment groups with respect to DOPAC concentration ($^*p < 0.05$) and no differences between treatment groups with respect to HVA concentration.

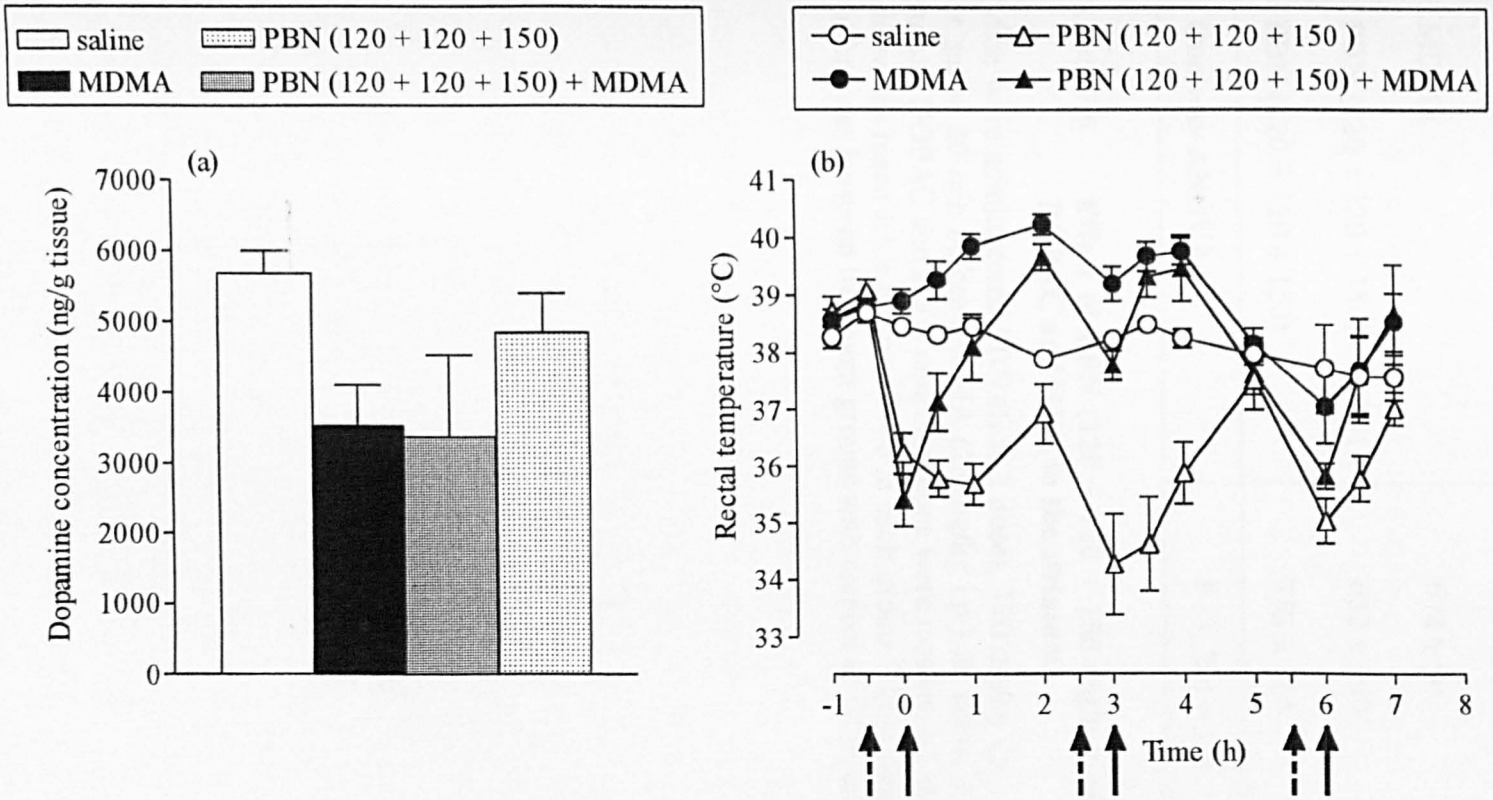

Figure 7.12 Effect of α-phenyl-*N*-tert-butyl nitrone (PBN, 120 + 120 + 150 mg/kg) on MDMA-induced striatal dopamine loss and acute hyperthermia.

Mice were administered PBN (first 2 doses, 120 mg/kg i.p., third dose, 150 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals. Results shown as mean ± s.e.m., n = 3 – 6 in each group. ***(a) Striatal dopamine concentration measured 1 week after treatment.*** There were no statistically significant differences between treatment groups. ***(b) MDMA-induced acute hyperthermia.*** Broken arrows denote times of PBN/saline injections, full arrows denote MDMA/saline injections. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in rectal temperature ($F(1, 10) = 9.0$, $p < 0.05$) compared to saline-treated mice. PBN induced a hypothermic response in both MDMA-treated ($F(1, 8) = 10.8$, $p < 0.05$) and saline-treated mice ($F(1, 10) = 27.6$, $p < 0.001$).

Treatment	Dopamine metabolite concentration (ng/g tissue) DOPAC	HVA
Saline	961 ± 68	574 ± 34
MDMA	678 ± 90	411 ± 39
PBN (120 + 120 + 150) + MDMA	632 ± 169	405 ± 76
PBN (120 + 120 + 150)	753 ± 114	468 ± 47
One-way ANOVA	F (3, 20) = 1.9	F (3, 20) = 2.9

Table 7.9 Effect of PBN (120 + 120 + 150 mg/kg) on MDMA-induced loss of DOPAC and HVA in the striatum.

Mice were administered PBN (first 2 doses, 120 mg/kg i.p., third dose, 150 mg/kg i.p.) or saline 30 min before MDMA (25 mg/kg i.p.) or saline, 3 times at 3 h intervals, and striatal DOPAC and HVA concentrations were measured 1 week post-treatment. Results shown as mean ± s.e.m., n = 3 – 6 in each group. There were no statistically significant differences between treatment groups with respect to DOPAC or HVA concentrations.

7.4 DISCUSSION

This study has highlighted some of the differences in MDMA-induced neurotoxicity between rats and mice and has demonstrated that putative neuroprotective compounds differ in their ability to prevent long-lasting damage to monoaminergic systems in the two species. In concurrence with previous studies (Laverty & Logan, 1990; Logan *et al.*, 1988; Miller & O'Callaghan, 1994; 1995; O'Callaghan & Miller, 1994; O'Shea *et al.*, 2001), MDMA administration produced a selective neurotoxic loss of striatal dopamine and its metabolites, while regional brain concentrations of 5-HT and 5-HIAA were unaffected. In addition, none of the pretreatment compounds employed had any effect on 5-HT or 5-HIAA concentrations.

Previous studies on the effects of both methamphetamine and MDMA in mice have demonstrated that several doses (three or four doses administered within 24 h) are required to produce neurotoxic damage (e.g. Colado *et al.*, 1999c; Laverty & Logan, 1990; Logan *et al.*, 1988; Miller & O'Callaghan, 1993; 1994; 1995; O'Callaghan & Miller, 1994; O'Shea *et al.*, 2001; Sonsalla *et al.*, 1989; 1991; Stone *et al.*, 1987a). The effects of one, two or three doses of MDMA on striatal monoamine concentrations were thus investigated in the current study. 5-HT and 5-HIAA concentrations were unaltered, whereas dose-dependent reductions in dopamine, DOPAC and HVA were observed: three doses of MDMA administered at 3 h intervals resulted in a 71 % reduction in striatal dopamine concentration compared to control animals, two doses induced a 57 % reduction, and one dose had no significant effect on striatal dopamine concentration. All subsequent experiments therefore utilised the administration of three doses of MDMA at 3 h intervals and resulted in 40 - 60 % reductions in striatal dopamine concentration in MDMA-treated mice.

In the preliminary experiment, the temperature responses after one, two and three doses of MDMA were also monitored. A single dose produced a rise in rectal temperature of approximately +1.5 °C, which was sustained for over 3 h; two doses resulted in a temperature rise after the first dose, which was sustained for over 5 h; three doses resulted in a hyperthermic response after the first dose, which was sustained for over 5 h, temperature then declined towards control values prior to the third dose, after which

an increase in temperature was observed. In all other experiments, an increase in rectal temperature of approximately +2 °C was observed following the first dose of MDMA and rectal temperature declined towards pretreatment values prior to the second dose, after which a second rise in temperature was observed. The same pattern of decline towards control values and subsequent hyperthermic response were seen prior to and following the third dose of MDMA, respectively. The likely reason for the different temperature responses seen in the preliminary experiment, compared to all other experiments, is the different thermometer used - a digital thermometer was used in the preliminary experiment, which gave lower value readings than the rectal probes used in all other experiments.

The two NMDA antagonists used in the current study - AR-R15896AR and MK-801 - have previously been demonstrated to be neuroprotective in animal models of ischaemia (see Buchan & Pulsinelli, 1990; Cregan *et al.*, 1997). MK-801 has also been shown to protect against MDMA-, methamphetamine- and *p*-chloroamphetamine-induced neurotoxicity in rats and against MDMA-, MDA- and methamphetamine-induced neurotoxicity in mice (as reviewed in the introduction; see section 7.1.2). However, MK-801 has also been shown to induce hypothermia, which itself is likely to produce neuroprotection (Albers & Sonsalla, 1995; Ali *et al.*, 1994; Farfel & Seiden, 1995a; 1995b; Miller & O'Callaghan, 1994; 1995). In the current study, neither AR-R15896AR nor MK-801 protected against MDMA-induced dopaminergic neurotoxicity in mice; the lack of protective effect of AR-R15896AR concurred with results seen in rats, where it did not protect against MDMA-induced serotonergic damage (Colado *et al.*, 1998). In addition, neither of the NMDA antagonists had any effect on MDMA-induced hyperthermia, with the exception of the third dose in each case; both AR-R15896AR and MK-801 attenuated the hyperthermic response to MDMA treatment following the third dose of pretreatment compound. However, since neither of the NMDA antagonists prevented MDMA-induced neurotoxicity, it seems likely that the degree of hypothermia produced following the third dose of antagonist in each experiment was insufficient to provide neuroprotection. Thus it appears that activation of NMDA receptors is not directly involved in MDMA-induced neurotoxicity in mice.

The GABAmimetic compound clomethiazole has been demonstrated to provide neuroprotection in animal models of ischaemia and against MDMA-induced neurotoxicity in rats and methamphetamine-induced neurotoxicity in rats and mice (see section 7.1.2). However, clomethiazole did not alter MDMA-induced striatal dopamine loss in mice and, with the exception of the third dose, clomethiazole also did not modify MDMA-induced hyperthermia. Therefore, the fact that clomethiazole is neuroprotective against MDMA-induced serotonergic neurotoxicity in rats (although the mechanism involved is yet to be clarified), the lack of neuroprotection in mice indicates some different mechanism of action between the two species.

Each of the three NOS inhibitors used in the current study (AR-R17477AR, S-MTC and 7-NI) provided significant neuroprotection against MDMA-induced dopamine loss in the mouse striatum. These results are, in general, in agreement with previous studies where all three compounds have been demonstrated to provide neuroprotection in different rodent models of neurodegeneration. For example, AR-R17477AR provides protection in animal models of global and focal ischaemia, S-MTC and 7-NI protect against methamphetamine-induced neurotoxicity in mice, and 7-NI protects against MDMA-induced neurotoxicity in mice (see section 7.1.2). However, there is some controversy as to whether 7-NI is neuroprotective through effects on amphetamine-induced hyperthermia; Callahan & Ricaurte (1998) reported that 7-NI prevented methamphetamine-induced hyperthermia, and Colado *et al.* (1999c) demonstrated similar effects following MDMA administration to mice. In contrast, Di Monte *et al.* (1996), Itzhak & Ali (1996) and Itzhak *et al.* (2000) did not demonstrate any effects of 7-NI on methamphetamine-induced hyperthermia, except at a high dose (50 mg/kg administered 30 min before methamphetamine, three times at 3 h intervals; Itzhak *et al.*, 2000). In the current study, AR-R17477AR only attenuated MDMA-induced hyperthermia after the third dose. Based on the NMDA antagonist experiments, where hyperthermia was also only attenuated after the third dose of pretreatment compound (see section 7.3.2), this should not, in itself, have provided neuroprotection. S-MTC did not modify the MDMA-induced hyperthermic response, but did provide significant neuroprotection, while 7-NI did prevent MDMA-induced hyperthermia. Therefore, in mice, NOS inhibitors have been demonstrated to play an important role in protection

against MDMA-induced striatal dopamine loss, which, in the case of AR-R17477AR and S-MTC, is not dependent on modification of the hyperthermic response.

In the current study, PBN only provided neuroprotection against MDMA-induced striatal dopamine loss where the acute hyperthermic response was significantly attenuated or completely prevented: (1) the lowest dose administered (120 mg/kg) did not modify MDMA-induced reductions in striatal dopamine measured one week post-treatment and had no effect on MDMA-induced hyperthermia; (2) administration of the highest dose (150 mg/kg) resulted in striatal dopamine concentrations in PBN + MDMA-treated mice being the same as those in saline-treated mice, but a hypothermic response was observed in PBN-treated mice; (3) the intermediate dose of PBN (120 + 120 + 150 mg/kg) did not prevent MDMA-induced striatal dopamine loss and did not alter the peak hyperthermic responses of MDMA-treated mice. These results are consistent with previous studies, which have demonstrated PBN-induced neuroprotection against MDMA- and PCA-induced neurotoxicity in rats and against MPTP- and methamphetamine-induced neurotoxicity in mice, while there is some evidence for prevention of amphetamine-induced hyperthermia (as reviewed in the introduction; see section 7.1.2). The lack of protective effect of PBN against MDMA-induced neurotoxicity in mice indicates that the free radical species scavenged by PBN are not directly involved.

It should be noted here that rectal temperature was measured in all experiments, thus hyperthermic and hypothermic responses are described with reference to rectal temperature rather than brain temperature. Neuroprotection can be attained in animal models of ischaemia by reducing brain temperature (e.g. Barone *et al.*, 1997), while rectal temperature has been reported to unreliably reflect brain temperature in such models (see Busto *et al.*, 1987). Δ9-tetrahydrocannabinol (THC) has also been reported to provide neuroprotection against focal and global ischaemia, possibly via the induction of hypothermia. However, chronic administration of THC has recently been demonstrated to induce an increase in brain temperature, while rectal temperature was not significantly altered (Perron *et al.*, 2001). The question therefore arises as to whether compounds which have been shown to be neuroprotective, such as AR-

R17477AR, provide such protection via a reduction in brain temperature (while rectal temperature is unaltered) or some other mechanism. Further examination of the mechanisms involved in mediation of brain and rectal temperature are clearly required and, ideally, measurement of both should be performed during investigation of putative neuroprotective compounds.

The current study has demonstrated that NOS is involved in MDMA-induced dopaminergic neurotoxicity in mice, but the exact mechanistic pathway is, as yet, unclear. There is believed to be an excitotoxic cascade of events which occurs following glutamatergic stimulation of NMDA receptors, resulting in the production of nitric oxide (NO) and free radicals (see Figure 7.13; Rang *et al.*, 1995). NMDA receptor activation results in increased calcium ion (Ca^{2+}) movement inwards through the NMDA receptor channel. Subsequently, calcium-calmodulin activates NOS, leading to the formation of NO + L-citrulline from L-arginine + oxygen (O_2). Depending on the redox state of the cell, NO may be formed as either: (1) $NO^{\bullet}$, which reacts with superoxide anions (O_2^-) to form peroxynitrite radicals ($ONOO^-$) or, (2) NO^+, which nitrosylates a sulphydryl group on the NMDA receptor causing its downregulation thus preventing further Ca^{2+} entry. $ONOO^-$ radicals can, in turn, cause lipid peroxidation or react with macromolecules, resulting in intracellular calcium overload and cell death. Superoxide dismutase (SOD) can prevent the formation of $ONOO^-$ radicals by causing the reduction of O_2^- to O_2 + hydrogen peroxide (H_2O_2), which is further degraded to O_2 + water (H_2O) via the action of catalase. $NO^{\bullet}$ also activates guanylate cyclase, which leads to the production of cyclic guanosine monophosphate (cGMP), having subsequent actions on protein kinases and ion channels (see Garthwaite *et al.*, 1989; Moncada *et al.*, 1991; Rang *et al.*, 1995; Wiesinger, 2001).

The formation of $NO^{\bullet}$ and, subsequently, $ONOO^-$, is believed to be involved in methamphetamine-induced dopaminergic neurotoxicity in mice. For example, Di Monte *et al.* (1996) proposed a pathway whereby oxygen free radicals (e.g. O_2^-) are produced following methamphetamine-induced dopamine release, and interact with $NO^{\bullet}$ (produced via a glutamate-activated NOS pathway) to produce $ONOO^-$ radicals (Di Monte *et al.*, 1996). Further evidence for the production of $ONOO^-$ following

methamphetamine administration involves the formation of 3-nitrotyrosine (3-NT), which is an *in vivo* marker of $ONOO^-$ generation (Imam & Ali, 2000; Imam *et al.*, 1999; 2001). Peroxynitrite production and methamphetamine-induced dopaminergic neurotoxicity can be prevented by pretreatment with a selective $ONOO^-$ decomposition catalyst (5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron III; FeTMPyP), however this compound also prevents methamphetamine-induced hyperthermia (Imam *et al.*, 1999). Selenium, an antioxidant compound, has also been demonstrated to attenuate methamphetamine-induced $ONOO^-$ production in both *in vivo* and *in vitro* systems (Imam & Ali, 2000).

Nitric oxide has similarly been demonstrated to be involved in the long-term effects of MDMA in rats, as shown by the protective effects of NOS inhibitors. For example, Taraska & Finnegan (1997) administered MDMA or methamphetamine to SD rats (10 mg/kg, four times at 2 h intervals) and demonstrated significant attenuation of MDMA-induced serotonergic loss and methamphetamine-induced dopaminergic loss, where animals were pretreated with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). However, the protective effects of L-NAME against methamphetamine-induced dopamine loss, at least, were accompanied by the prevention of methamphetamine-induced hyperthermia (Taraska & Finnegan, 1997). Zheng & Laverty (1998) demonstrated partial neuroprotection by the NOS inhibitor N^{ω}-nitro-L-arginine (L-NOARG) against MDMA-induced serotonin depletion in rat frontal and parietal cortex seven days post-treatment, while acute hyperthermia was unaltered.

The involvement of NO in amphetamine-induced neurotoxicity has also been demonstrated *in vitro*. For example, Cerruti *et al.*, (1995) demonstrated that methamphetamine and MDMA were toxic to embryonic rat brain dopaminergic and serotonergic cells, respectively, and that NOS inhibitors (L-N^G-nitro-arginine (*N*-Arg), L-nitro-methylarginine (L-NMA) and aminoguanidine) protected against this toxicity.

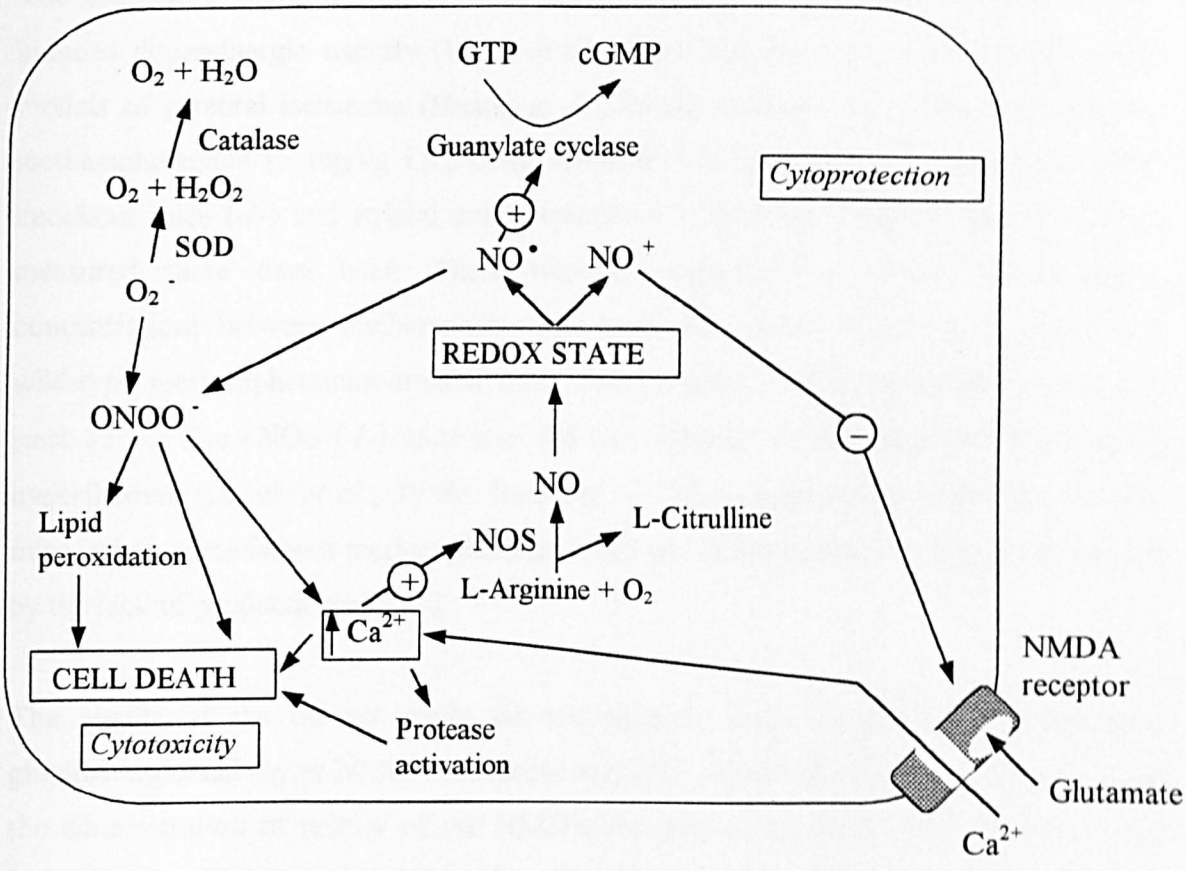

Figure 7.13 Postulated mechanism for the induction of cytotoxicity or cytoprotection through the formation of nitric oxide (NO) (after Rang *et al.*, 1995).

Glutamatergic stimulation of NMDA receptors results in increased calcium (Ca^{2+}) entry through the receptor channel. Calcium-calmodulin activates nitric oxide synthase (NOS), leading to the formation of NO, as either: (1) $NO^\bullet$, which reacts with superoxide anions (O_2^-) to form peroxynitrite radicals ($ONOO^-$), leading to lipid peroxidation, intracellular calcium overload and cell death, or (2) NO^+, which nitrosylates a sulphydryl group on the NMDA receptor causing its downregulation and prevention of further Ca^{2+} entry. Superoxide dismutase (SOD) can prevent the formation of $ONOO^-$ by causing the reduction of O_2^- to oxygen (O_2) + hydrogen peroxide (H_2O_2), which is further degraded to O_2 + water (H_2O) via the action of catalase. $NO^\bullet$ also activates guanylate cyclase, leading to the production of cyclic guanosine monophosphate (cGMP).

The neuronal isoform of NOS (nNOS) is believed to be involved in methamphetamine-induced dopaminergic toxicity (Imam *et al.*, 2001; Itzhak *et al.*, 1998) and in animal models of cerebral ischaemia (Huang *et al.*, 1994). Itzhak *et al.* (1998) administered methamphetamine (5 mg/kg i.p., three times at 3 h intervals) to homozygote nNOS knockout mice (-/-) and striatal concentrations of dopamine, DOPAC and HVA were measured three days later. There was no difference in striatal catecholamine concentrations between methamphetamine- and saline-treated nNOS (-/-) mice, while wild-type methamphetamine-treated mice demonstrated striatal dopamine losses of at least 35 %. The nNOS (-/-) mice also did not demonstrate methamphetamine-induced hyperthermia (Itzhak *et al.*, 1998). Imam *et al.* (2001) demonstrated that nNOS (-/-) mice, when administered methamphetamine, did not produce peroxynitrite as evidenced by the lack of production of 3-NT.

The results of the current study do not support direct involvement of increased glutamatergic activity at NMDA receptors in MDMA-induced striatal dopamine loss, as the administration of neither of the NMDA receptor antagonists (AR-R15896AR and MK-801) provided any neuroprotection. However, as two of the NOS inhibitors used (AR-R17477AR and S-MTC) did provide substantial neuroprotection, it may be that some mechanism other than NMDA receptor stimulation triggers NOS activation. Although PBN was only neuroprotective where hyperthermia was prevented, there is evidence to support the involvement of free radicals in MDMA-induced neurotoxicity in mice (Cadet *et al.*, 1994; 1995; 2001; Colado *et al.*, 2001; Jayanthi *et al.*, 1999). Colado *et al.* (2001) used an *in vivo* free radical-generating system to measure MDMA-induced free radical formation. The interaction of salicylic acid with hydroxyl radicals ($OH^{\bullet}$) (or peroxynitrite; Narayan *et al.*, 1997) generates 2,3- and 2,5-dihydroxybenzoic acid (2,3- and 2,5-DHBA), a reaction which is used both *in vivo* and *in vitro* as an assay for measuring $OH^{\bullet}$ and $ONOO^{-}$ formation (see Chiueh *et al.*, 1992; 1993; Colado *et al.*, 1997a; 1999a; 2001; Giovanni *et al.*, 1995; Halliwell & Kaur, 1997; Narayan *et al.*, 1997). For *in vivo* measurement, a microdialysis probe is implanted in the brain and perfused with salicylic acid, the resulting dialysate is analysed via h.p.l.c. and the concentration of 2,3- and 2,5-DHBA present thus provides a measure of $OH^{\bullet}$ production (see Chiueh *et al.*, 1992; 1993; Colado *et al.*, 1997a; 2001). Colado *et al.*

(2001) demonstrated that MDMA administration to mice (20 mg/kg, three times at 3 h intervals) resulted in a significant increase in the conversion of salicylic acid to 2,3-DHBA in the striatal dialysate, indicating MDMA-induced free radical formation. This result is in agreement with studies performed in rats, where MDMA administration leads to increased conversion of salicylate to 2,3-DHBA (Colado *et al.*, 1997a; 1999a).

Free radical involvement in MDMA-induced neurotoxicity has also been demonstrated in both *in vivo* and *in vitro* copper/zinc superoxide dismutase (Cu/ZnSOD) systems: (1) homozygous and heterozygous transgenic mice carrying the human Cu/ZnSOD gene have shown significant attenuation of MDMA-induced reductions in striatal dopamine concentration, indicating a role for oxygen-based free radicals in MDMA-induced neurotoxicity (Cadet *et al.*, 1994; 1995; 2001; Jayanthi, 1999); (2) embryonic rat brain cell cultures carrying the human Cu/ZnSOD enzyme are resistant to both methamphetamine- and MDMA- induced toxicity (Cerruti *et al.*, 1995). MDMA administration has also been shown to cause significant decreases in SOD, catalase and glutathione peroxidase activity, and significant increases in lipid peroxidation, in the brains of non-transgenic mice. Homozygous Cu/ZnSOD mice, however, are unaffected by MDMA with regard to SOD and catalase activity and lipid peroxidation, while glutathione peroxidase activity is increased (Cadet *et al.*, 2001).

In addition to glutamate-activated production of free radicals, dopamine released by MDMA may be auto-oxidised, resulting in the production of $OH^{\bullet}$ and quinones (see Chiueh *et al.*, 1993; Graham *et al.*, 1978), the latter also being formed as a product of MDMA metabolism (Hiramatsu *et al.*, 1990). Cytotoxicity may be induced by disruption of mitochondrial electron transport systems, through the actions of oxygen-based free radicals and dopamine itself (Ben-Shachar *et al.*, 1995; Zhang *et al.*, 1990). Using the *in vivo* microdialysis system for measurement of $OH^{\bullet}$ formation (see above), Colado *et al.* (2001) demonstrated that AR-R17477AR prevented MDMA-induced free radical production. The NOS inhibitor (5 mg/kg) was administered 30 min prior to each dose of MDMA (20 mg/kg, three times at 3 h intervals) and abolished MDMA-induced increases in 2,3-DHBA formation, thus indicating prevention of free radical production. Furthermore, the inhibitory actions of AR-R17477AR were demonstrated not to involve

trapping of free radicals, through its lack of effect on *in vitro* lipid peroxidation. Incubation of rat cortical synaptosomes with ferrous chloride ($FeCl_2$) and ascorbic acid results in production of malondialdehyde (a thiobarbituric acid-reacting substance (TBARS), derived from the breakdown of polyunsaturated fatty acids), which is quantified by a 2-thiobarbituric acid colour reaction (see Colado *et al.*, 2001; Das & Ratty, 1987). In contrast to the free radical scavenger butylated hydroxytoluene (BHT), AR-R17477AR had no effect on malondialdehyde formation measured in this manner, indicating that it does not act as a free radical scavenger (Colado *et al.*, 2001).

Since the neuronal isoform of NOS is believed to be involved in methamphetamine-induced neurotoxicity, the selectivity of the NOS inhibitors which protected against MDMA-induced striatal dopamine loss in the current study, is also likely to be important. S-MTC has been demonstrated to be a potent, reversible, slow-binding inhibitor of all of the human NOS isoforms - nNOS, inducible NOS (iNOS) and endothelial NOS (eNOS) - its effects on nNOS being the most potent (Furfine *et al.*, 1994). In addition, S-MTC has been shown to inhibit rat nNOS and iNOS (Narayanan & Griffith, 1994). AR-R17477AR has also been shown to be a potent inhibitor of human nNOS, being 17-fold selective for nNOS versus eNOS (O'Neill *et al.*, 2000). Although further studies need to be performed, to assess the selectivity of these compounds for nNOS versus the other NOS isoforms in rodents, it does appear that nNOS is involved in MDMA-induced neurotoxicity.

In conclusion, this study has confirmed that MDMA administration to mice results in selective dopaminergic toxicity, as shown by striatal loss of dopamine and its metabolites. Furthermore, the neuroprotective effects of preventing MDMA-induced hyperthermia have been demonstrated - in particular, neuroprotection afforded by 7-NI and PBN could not be separated from modification of the acute hyperthermic response. In the case of the GABAmimetic compound clomethiazole, it appears that some different mechanism of action may exist between rats and mice; clomethiazole is neuroprotective against MDMA-induced serotonergic damage in rats, but did not have any effect on the striatal dopamine loss in mice. The two compounds which significantly attenuated MDMA-induced reductions in striatal dopamine concentration

without modifying the acute hyperthermic response - AR-R17477AR and S-MTC - are believed to be relatively selective nNOS inhibitors, thus indicating the involvement of nNOS in the neurotoxic pathway. Although PBN did not provide neuroprotection, there is evidence that free radicals are being formed and their production is inhibited by AR-R17477AR. As neither of the NMDA antagonists (AR-R15896AR and MK-801) were neuroprotective, free radicals are presumably not being formed as a result of excessive glutamatergic activity at NMDA receptors, but by some other route - probably via the auto-oxidation of MDMA metabolites (see Figure 7.14).

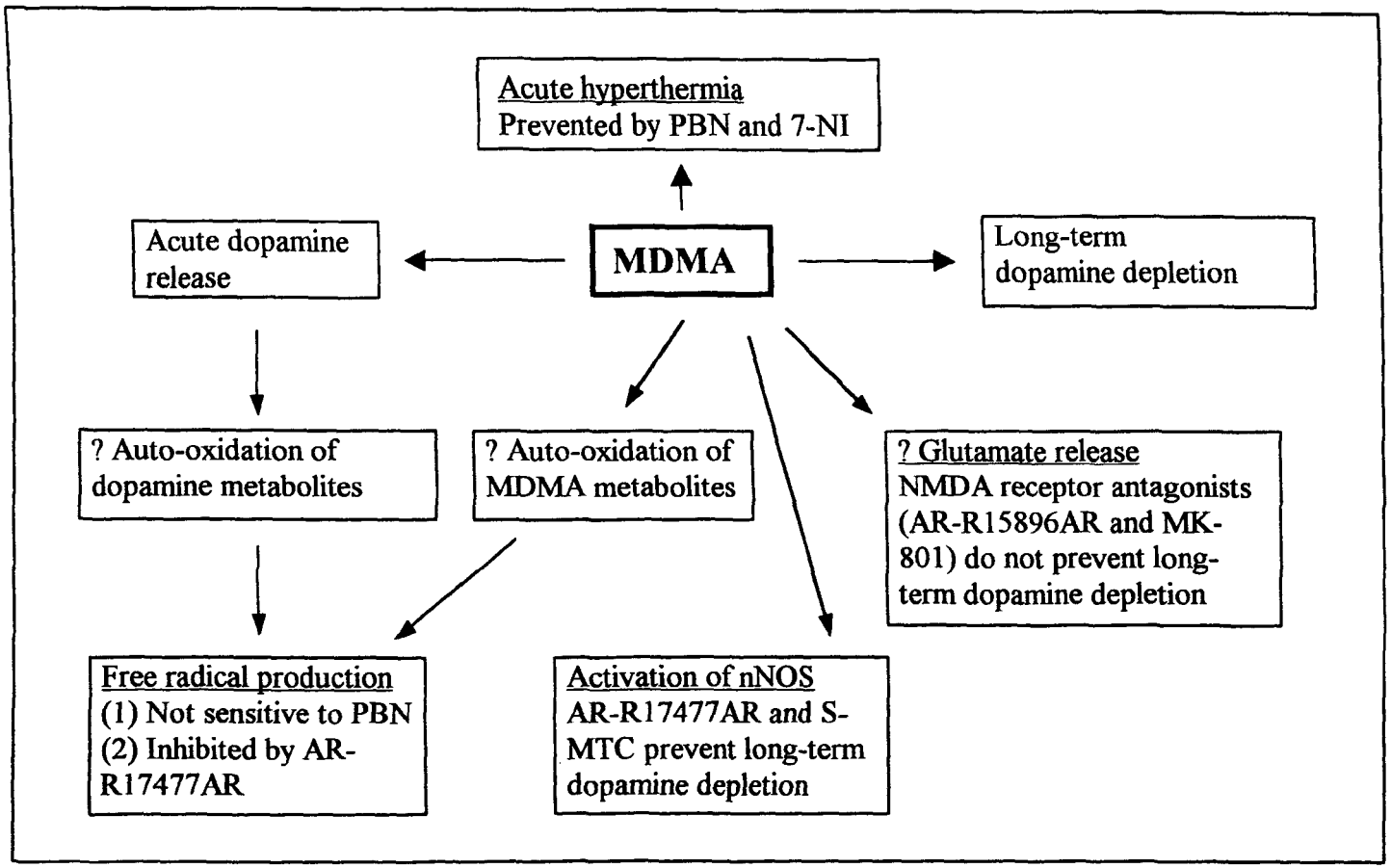

Figure 7.14 Schematic representation of putative neurotoxic mechanisms following MDMA administration to mice.

MDMA results in an acute release of dopamine and long-term dopamine depletion in the striatum, in addition to an acute hyperthermic response. The lack of neuroprotection afforded by the NMDA antagonists, AR-R15896AR and MK-801, indicates that glutamatergic activation of NMDA receptors is not directly involved in MDMA-induced neurotoxicity in mice. The relatively selective nNOS inhibitors, AR-R17477AR and S-MTC, significantly attenuate striatal dopamine depletion, without altering the hyperthermic response, indicating the involvement of nNOS in the neurotoxic pathway. 7-NI only provides neuroprotection where acute hyperthermia is prevented. Free radicals are produced and inhibited by AR-R17477AR (as shown via *in vivo* microdialysis), but the free radical scavenger PBN only provides neuroprotection where the acute hyperthermic response is prevented.

CHAPTER 8

GENERAL DISCUSSION

8 GENERAL DISCUSSION

The aims of this study were to examine some of the acute and long-term functional consequences of MDMA administration to rats. Studies of the long-term behavioural and thermoregulatory responses were conducted, and a pharmacological investigation was made of the acute MDMA-induced hyperthermic response. In addition, the mechanisms involved in the neurotoxic effects of MDMA administration to mice were investigated.

8.1 BASELINE ANXIETY-RELATED AND LOCOMOTOR BEHAVIOUR OF DARK AGOUTI RATS

Prior to investigation of the long-term behavioural consequences of a single neurotoxic dose of MDMA in Dark Agouti (DA) rats, a pilot study was performed to enable determination of the baseline anxiety-related and locomotor behaviour of this strain. Thus the behaviour of DA rats was compared with that of a more commonly used strain, the Sprague-Dawley (SD), on the elevated plus-maze and in the open-field. DA rats showed significantly higher levels of anxiety-related behaviour, as evidenced by lower open arm behaviour (% open arm entries and % time spent on the open arms) on the plus-maze and fewer crossings towards the centre of the open-field, compared to the SD strain. In addition, DA rats demonstrated significantly lower levels of locomotor activity, as indicated by lower numbers of total and closed arm entries on the plus-maze and total zone crossings in the open-field. Ethological variables, such as defecation and grooming (which were markedly greater in DA rats), provided further evidence of increased "anxiety" in this strain.

Subsequently, the responses of both strains to acute diazepam administration were assessed on the elevated plus-maze. However, the differences between the two strains were abolished following acute handling whilst injecting vehicle. While both strains demonstrated an anxiolytic response to diazepam, shown by increased % time spent on the open arms, vehicle-treated DA rats were no longer different in their responses compared to vehicle-treated SD rats. For example, the % number of open arm entries and % time spent on the open arms by vehicle-treated DA rats were approximately

three-fold higher than the values recorded in naïve DA rats, which resulted in similar % open arm entries and % time spent on the open arms in both vehicle-treated strains.

Although the magnitude of anxiety-related responses was greater in naïve DA rats compared to the SD strain, responses were still qualitatively similar. Thus DA rats were deemed suitable for measurement of "anxiety"- and locomotor- behaviour in MDMA-treated animals.

8.2 LONG-TERM BEHAVIOURAL CONSEQUENCES OF MDMA ADMINISTRATION

The long-term behavioural consequences of a single neurotoxic dose of MDMA (12.5 mg/kg i.p.), shown to produce a 20 - 40 % depletion of 5-HT, were investigated in DA rats. Subjects were tested on the elevated plus-maze and in the open-field at three time-points: (1) one week post-treatment (Days 8 - 11), (2) one month post-treatment (Days 29 - 32), and (3) ten to eleven weeks post-treatment (open-field: Days 71 - 73; plus-maze: Day 80). Significant differences in anxiety-related- and locomotor- behaviour were observed in both "anxiety" models. For example, on Day 80 MDMA-pretreated rats spent a greater % time on the open arms of the plus-maze, and on Day 73 performed a greater, although not statistically significant, number of Zone 2 crossings in the open-field. The open arms of the plus-maze are believed to evoke a "fear" response (see Montgomery, 1955; Handley & Mithani, 1984), while crossings towards the centre of the open-field involves the conflict between exploratory drive and avoidance of open, brightly lit spaces (see Schmitt & Hiemke, 1998a). Thus these data strongly indicated that a single dose of MDMA, ten to eleven weeks earlier, produced a marked reduction in "anxiety". In addition, MDMA-pretreated rats demonstrated higher levels of locomotor activity on the plus-maze, shown by greater numbers of total and closed arm entries on all three test days compared to control animals, total arm entries reaching statistical significance on Day 80. Similar results were observed in the open-field, at least under red-light illumination, where MDMA-pretreated rats performed greater numbers of total crossings on each test day which again reached statistical significance at the latest time-point.

Disruption of serotonergic activity induced by 5,7-DHT (see Briley *et al.*, 1990; Hall *et al.*, 1999; Tye *et al.*, 1977) or PCPA (see File & Hyde, 1977; Tye *et al.*, 1979) results in reduced anxiety-like behaviour, thus it could be expected that MDMA-induced serotonergic neurotoxicity would have similar consequences. While there is some evidence of increased anxiety in human recreational users of MDMA, it is difficult to separate the effects of MDMA from those of other illicit drugs or from any pre-existing psychological conditions (see Parrott *et al.*, 2000). However, McCann *et al.*, (1999) demonstrated that chronic users of MDMA were less susceptible to an *m*-CPP-induced panic attack than non-MDMA users, which was suggested to indicate a downregulation of postsynaptic 5-HT_{2C} receptors and could be indicative of functional consequences of MDMA ingestion. In addition, the reduction in "anxiety" seen in rats in the current study could be interpreted as an increase in "impulsivity", which has been reported to occur in human recreational users of MDMA (Morgan, 1998).

The fact that marked reductions in anxiety-related behaviour did not become apparent until over two months after a single dose of MDMA, while significant serotonergic depletions have been observed one week post-treatment (see Colado *et al.*, 1995; O'Shea *et al.*, 1998), indicates that the behavioural changes observed are not solely due to serotonergic neurotoxicity, but that adaptive changes are occurring within the brain.

8.3 LONG-TERM CONSEQUENCES OF MDMA ADMINISTRATION ON THERMOREGULATORY BEHAVIOUR

Previously reported studies have shown that: (1) a prior neurotoxic dose regimen of MDMA results in an attenuation of the acute hyperthermic response to a single dose of MDMA several weeks later (Shankaran & Gudelsky, 1999), and (2) prior multiple doses of MDMA resulted in prolonged hyperthermia when animals were subjected to a "thermoregulatory challenge" over three months later (Dafters & Lynch, 1998). In addition, conflicting results have been reported concerning the effects of prior MDMA administration on 8-OH-DPAT-induced hypothermia. Aguirre *et al.* (1998) demonstrated a potentiation of the hypothermic response in MDMA-pretreated animals, whereas McNamara *et al.* (1995) demonstrated no effect of MDMA-pretreatment.

Thus the current study was performed in an attempt to further elucidate the long-term consequences of MDMA pretreatment on thermoregulatory responses:

(1) Five to six weeks after a single neurotoxic dose of MDMA (12.5 mg/kg i.p.), DA rats were subjected to a high ambient temperature (T_a) "thermoregulatory challenge", which consisted of a 60 min exposure to a T_a of 30 ± 0.5 °C. Rectal temperature was monitored prior to, during, and after exposure to the high or low T_a environment. Prior to the challenge, there was no difference in rectal temperature values between MDMA- and saline-pretreated animals. However, rats which had been administered MDMA five to six weeks earlier demonstrated a faster rise in rectal temperature during the challenge and a sustained hyperthermic response upon being returned to a 'normal' environment (20 ± 2 °C). These data indicated a long-term defect in thermoregulation, were consistent with the findings of Dafters & Lynch (1998), and were accompanied by 20 - 40 % depletions in regional brain concentrations of 5-HT and 5-HIAA, as measured one week after MDMA administration.

(2) A second challenge was performed where animals were subjected to a low T_a (10 ± 0.5 °C) for 1 h, five to six weeks after administration of MDMA (12.5 mg/kg i.p.). In this experiment the rectal temperature of MDMA-pretreated rats remained marginally higher than control values during and after the challenge, which could indicate a lack of effect of MDMA on heat-retaining mechanisms. However, since animals were group-housed (three per cage), heat retention could have been achieved by the rats huddling together, and perhaps greater differences between treatment groups would have been observed if the animals had been housed separately.

(3) Another group of rats were administered one, two or three doses of MDMA (12.5 mg/kg i.p.) two or three weeks apart and the temperature response to each dose was monitored. There was no difference in the acute hyperthermic response between animals which had been administered one or two previous doses of MDMA and those animals which had only received a single dose. These data showed that a 20 - 40 % depletion of 5-HT had no long-lasting effect on the acute hyperthermic response, which was in contrast to the results of Shankaran & Gudelsky (1999) who reported that a neurotoxic dose regimen of MDMA attenuated the acute hyperthermia induced by a single dose one week later. However, the differences in experimental methodology

between the current study and that of Shankaran & Gudelsky (1999) make a direct comparison of results unfeasible. It is possible that only a more severe loss of 5-HT content (e.g. 70 - 80 %) would have significant, long-lasting effects on the MDMA-induced acute hyperthermic response.

(4) Rats which had been injected with MDMA (12.5 mg/kg i.p.) three or four weeks previously were administered 8-OH-DPAT (0.057, 0.09, 0.11 mg/kg s.c.) and their temperature response monitored. There was no significant difference in the acute hypothermic response to 8-OH-DPAT, at any of the doses administered, between MDMA- and saline-pretreated animals. These results were consistent with those of McNamara *et al.* (1995), indicating that MDMA administration has no lasting effect on 5-HT_{1A} receptor function.

Thus the current study indicated long-term functional consequences of MDMA administration, and a 20 - 40 % depletion of regional brain 5-HT, on thermoregulatory responses to a high T_a challenge. However, this degree of loss of 5-HT content was insufficient to alter subsequent hyperthermic responses to acute MDMA administration and did not alter 5-HT_{1A} receptor function.

8.4 AN INVESTIGATION OF THE ACUTE HYPERTHERMIC RESPONSE TO MDMA ADMINISTRATION

During the course of the studies on the longer-term thermoregulatory responses to MDMA administration, it was decided that the acute hyperthermic response required further investigation. MDMA administration results in an acute release of 5-HT (see Colado & Green, 1994; Crespi *et al.*, 1997; Johnson *et al.*, 1986; Schmidt *et al.*, 1987; Stone *et al.*, 1986), and the acute hyperthermic response which also follows MDMA (see Dafters, 1994; Malberg *et al.*, 1996; O'Shea *et al.*, 1998) has been assumed to be 5-HT-mediated (see Shankaran & Gudelsky, 1999). Dopamine is also acutely released following MDMA administration to rats (see Colado *et al.*, 1999a; Gudelsky *et al.*, 1994; Yamamoto & Spanos, 1988), and stimulation of 5-HT_2 receptors following MDMA-induced 5-HT release has been suggested to further enhance dopamine release, at least in the striatum (see Gudelsky *et al.*, 1994; Nash, 1990; Schmidt *et al.*, 1999b).

Thus a series of 5-HT and dopamine receptor antagonists and uptake inhibitors were employed in an attempt to determine the receptor subtype(s) involved in this response. Administration of the non-selective 5-$HT_{1/2}$ receptor antagonist, methysergide, the 5-HT_2 antagonist, ritanserin, or the 5-HT_{2C} antagonist, SB 242084, had no effect on the MDMA-induced hyperthermic response. The selective 5-HT_{2A} antagonist, MDL 100,907, modestly attenuated the response following the highest dose, while the selective 5-HT_2 antagonist, MDL 11,939, completely blocked the MDMA-induced acute hyperthermia. Neither of the selective serotonin uptake inhibitors, zimeldine or fluoxetine, had any effect on the hyperthermic response, even though fluoxetine almost completely abolished the MDMA-induced acute hippocampal release of 5-HT. The dopamine D_2 receptor antagonist, remoxipride, and the dopamine uptake inhibitor, GBR 12909, also had no effect on the MDMA-induced acute hyperthermic response, while the selective D_1 antagonist, SCH 23390, dose-dependently inhibited the response.

Therefore, with the exception of MDL 11,939 and the highest dose of MDL 100,907, these data strongly indicated that 5-HT is not directly involved in mediation of the acute hyperthermia which follows MDMA administration, and that dopamine D_1 receptors play some role in the response.

8.5 AN INVESTIGATION OF MDMA-INDUCED NEUROTOXICITY IN MICE

While MDMA administration to rats (see Aguirre *et al.*, 1998; Colado *et al.*, 1993; Farfel & Seiden, 1995a; Scanzello *et al.*, 1993), guinea-pigs (see Battaglia *et al.*, 1988a), non-human primates (see Ricaurte *et al.*, 1988c) and humans (see McCann *et al.*, 1994; 1998) results in long-term serotonergic neurotoxicity, dopaminergic depletion occurs following MDMA administration to mice (see Battaglia *et al.*, 1988a; Logan *et al.*, 1988). However, the mechanisms involved in dopaminergic neurotoxicity in mice have been little studied, thus a series of putative neuroprotective compounds were administered prior to MDMA and striatal levels of dopamine and its metabolites were analysed one week later. In addition, since a number of compounds are believed to provide neuroprotection via attenuation of the acute hyperthermic response (see Ali *et*

al., 1994; Callahan & Ricaurte, 1998, Miller & O'Callaghan, 1994), rectal temperature was closely monitored throughout each experiment.

Neither of the NMDA antagonists, AR-R15896AR and MK-801, provided any protection against MDMA-induced dopamine depletion. The lack of effect of AR-R15896AR is consistent with the lack of protection against MDMA-induced serotonergic neurotoxicity in rats (see Colado *et al.*, 1998), and, while MK-801 has been shown to be neuroprotective, it has also been shown to attenuate amphetamine derivative-induced hyperthermia (see Albers & Sonsalla, 1995; Miller & O'Callaghan, 1994). The GABAmimetic compound, clomethiazole, also did not afford any protection against MDMA-induced dopamine depletion, which is in contrast to its neuroprotective effects against MDMA-induced 5-HT depletion in rats (see Colado *et al.*, 1993; Colado & Green, 1994; Hewitt & Green, 1994). The free radical scavenger, PBN, only provided protection against dopaminergic neurotoxicity where the acute hyperthermic response was attenuated. This result is consistent with the effects seen in rats, where PBN provides protection against serotonergic depletion, but also attenuates hyperthermia (see Colado & Green, 1995). All three NOS inhibitors used in the current study, AR-R17477AR, S-MTC and 7-NI, provided significant protection against MDMA-induced dopamine depletion, and have all been demonstrated to be neuroprotective in different animals models of neurodegeneration (see Colado *et al.*, 1999c; Itzhak *et al.*, 2000; O'Neill *et al.*, 2000). While AR-R17477AR and S-MTC had no significant effect on the acute hyperthermic response, 7-NI completely prevented the response, which is consistent with previously reported results (Callahan & Ricaurte, 1998; Colado *et al.*, 1999c).

The protective effects of AR-R17477AR and S-MTC, which are both relatively selective nNOS inhibitors, thus indicates the involvement of nNOS in the neurotoxic pathway. While PBN did not protect against dopamine loss, there is evidence that free radicals are produced in mice following MDMA administration (see Colado *et al.*, 2001). However, since neither NMDA antagonist provided neuroprotection, it appears that free radicals are not being formed as a result of increased glutamatergic activity at NMDA receptors, but could involve the auto-oxidation of MDMA metabolites.

8.6 CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The studies which have been reported in this thesis have demonstrated that administration of a single dose of MDMA (12.5 mg/kg i.p.) to rats resulted in a 20 - 40 % regional brain depletion of 5-HT and 5-HIAA, which was shown to have long-term functional consequences: (1) reductions in anxiety-related behaviour over two months after a single neurotoxic dose, and (2) long-lasting alterations in thermoregulatory responses to a high T_a challenge five to six weeks post-drug administration. Furthermore, the selective dopaminergic depletion following MDMA administration to mice was suggested to involve nNOS, and it appeared that the free radicals which are acutely generated are not as a result of excessive glutamatergic activity at NMDA receptors, but probably involve auto-oxidation of MDMA metabolites.

Suggestions for future work include:

(1) Extending the time period for testing "anxiety" on the plus-maze and in the open-field - animals could be tested up to six or 12 months post-treatment, and regional brain 5-HT levels could be analysed in a parallel treatment group at each time-point to enable evaluation of changes in behaviour with reference to serotonergic depletion and recovery.

(2) Additional behavioural tests (such as delayed non-match to place and prepulse inhibition) could be performed at the same time-points employed in the current study and up to six or 12 months after drug administration, to further explore the behavioural consequences of a single neurotoxic dose.

(3) The acute hyperthermic response to MDMA could be investigated in primates in the same manner as that employed in rats, whereby a series of drugs which affect 5-HT or dopamine function are administered prior to MDMA. If, for example, the D_1 antagonist SCH 23390 attenuated the hyperthermic response in primates, it could also be administered after MDMA in an attempt to lower body temperature. This could provide a better understanding of the acute hyperthermic response in humans (which can have potentially fatal consequences).

(4) A simple method of investigating whether long-term changes in thermoregulatory behaviour also occur in human recreational users of MDMA, could be to place subjects in a high ambient temperature environment (e.g. 30 °C) for a limited time period (e.g. 1 - 2 h) and monitor body temperature.

REFERENCES

REFERENCES

AGUIRRE, N., GALBETE, J.L., LASHERAS, B. & DEL RÍO, J. (1995). Methylenedioxymethamphetamine induces opposite changes in central pre- and postsynaptic 5-HT_{1A} receptors in rats. *European Journal of Pharmacology*, **281,** 101-105.

AGUIRRE, N., BALLAZ, S., LASHERAS, B. & DEL RÍO, J. (1998). MDMA ('Ecstasy') enhances 5-HT_{1A} receptor density and 8-OH-DPAT-induced hypothermia: blockade by drugs preventing 5-hydroxytryptamine depletion. *European Journal of Pharmacology*, **346,** 181-188.

ALBERS, D.S. & SONSALLA, P.K. (1995). Methamphetamine-induced hyperthermia and dopaminergic neurotoxicity in mice: pharmacological profile of protective and nonprotective agents. *The Journal of Pharmacology and Experimental Therapeutics*, **275,** 1104-1114.

ALI, S.F., NEWPORT, G.D., HOLSON, R.R., SLIKKER JR, W. & BOWYER, J.F. (1994). Low environmental temperatures or pharmacologic agents that produce hypothermia decrease methamphetamine neurotoxicity in mice. *Brain Research*, **658,** 233-238.

ALI, S.F. & ITZHAK, Y. (1998). Effects of 7-nitroindazole, an NOS inhibitor on methamphetamine-induced dopaminergic and serotonergic neurotoxicity in mice. *Annals of The New York Academy of Sciences*, **844,** 122-130.

ANDREWS, N. & FILE, S.E. (1993). Handling history of rats modifies behavioural effects of drugs in the elevated plus-maze test of anxiety. *European Journal of Pharmacology*, **235,** 109-112.

AZMITIA, E.C., MURPHY, R.B. & WHITAKER-AZMITIA, P.M. (1990). MDMA (Ecstasy) effects on cultured serotonergic neurons: evidence for Ca^{2+}-dependent toxicity linked to release. *Brain Research*, **510,** 97-103.

BALDWIN, H.A., COLADO, M.I., MURRAY, T.K., DE SOUZA, R.J. & GREEN, A.R. (1993). Striatal dopamine release *in vivo* following neurotoxic doses of methamphetamine and effect of the neuroprotective drugs, chlormethiazole and dizocilpine. *British Journal of Pharmacology*, **108,** 590-596.

BALDWIN, H.A., WILLIAMS, J.L., SNARES, M., FERREIRA, T., CROSS, A.J. & GREEN, A.R. (1994). Attenuation by chlormethiazole administration of the rise in extracellular amino acids following focal ischaemia in the cerebral cortex of the rat. *British Journal of Pharmacology*, **112,** 188-194.

BARNES, N.M. & SHARP, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology*, **38,** 1083-1152.

BARONE, F.C., FEUERSTEIN, G.Z. & WHITE, R.F. (1997). Brain cooling during transient focal ischemia provides complete neuroprotection. *Neuroscience and Biobehavioral Reviews*, **21,** 31-44.

BARRETT, P.J. (1992). Ecstasy and dantrolene. *British Medical Journal*, **305,** 1225.

BARRETT, P.J. & TAYLOR, G.T. (1993). 'Ecstasy' ingestion: a case report of severe complications. *Journal of the Royal Society of Medicine*, **86,** 233-234.

BATTAGLIA, G., YEH, S.Y., O'HEARN, E., MOLLIVER, M.E., KUHAR, M.J. & DE SOUZA, E.B. (1987). 3,4-methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin terminals in rat brain: quantification of neurodegeneration by measurement of [3H]paroxetine-labeled serotonin uptake sites. *The Journal of Pharmacology and Experimental Therapeutics*, **242,** 911-916.

BATTAGLIA, G., YEH, S.Y. & DE SOUZA, E.B. (1988a). MDMA-induced neurotoxicity: parameters of degeneration and recovery of brain serotonin neurons. *Pharmacology Biochemistry and Behavior*, **29,** 269-274.

BATTAGLIA, G., BROOKS, B.P., KULSAKDINUN, C. & DE SOUZA, E.B. (1988b). Pharmacologic profile of MDMA (3,4-methylenedioxymethamphetamine) at various brain recognition sites. *European Journal of Pharmacology*, **149,** 159-163.

BATTAGLIA, G., SHARKEY, J., KUHAR, M.J. & DE SOUZA, E.B. (1991). Neuroanatomic specificity and time course of alterations in rat brain serotonergic pathways induced by MDMA (3,4-methylenedioxymethamphetamine): assessment using quantitative autoradiography. *Synapse*, **8,** 249-260.

BEN-SHACHAR, D., ZUK, R. & GLINKA, Y. (1995). Dopamine neurotoxicity: inhibition of mitochondrial respiration. *Journal of Neurochemistry*, **64,** 718-723.

BERGER, U.V., GU, X.F. & AZMITIA, E.C. (1992). The substituted amphetamines 3,4-methylenedioxymethamphetamine, methamphetamine, p-chloroamphetamine and fenfluramine induce 5-hydroxytryptamine release via a common mechanism blocked by fluoxetine and cocaine. *European Journal of Pharmacology*, **215,** 153-160.

BERTOGLIO, L.J. & CAROBREZ, A.P. (2000). Previous maze experience required to increase open arms avoidance in rats submitted to the elevated plus-maze model of anxiety. *Behavioural Brain Research*, **108,** 197 - 203.

BHATTACHARY, S. & POWELL, J.H. (2001). Recreational use of 3,4-methylenedioxymethamphetamine (MDMA) or 'ecstasy': evidence for cognitive impairment. *Psychological Medicine*, **31,** 647-658.

BISCHOFF, S., HEINRICH, M., SONNTAG, J.M. & KRAUSS, J. (1986). The D-1 dopamine receptor antagonist SCH 23390 also interacts potently with brain serotonin (5-HT_2) receptors. *European Journal of Pharmacology*, **129,** 367-370.

BOIREAU, A., BORDIER, F., DUBÉDAT, P. & DOBLE, A. (1995). Methamphetamine and dopamine neurotoxicity: differential effects of agents interfering with glutamatergic transmission. *Neuroscience Letters*, **195,** 9-12.

BOLLA, K.I., MCCANN, U.D. & RICAURTE, G.A. (1998). Memory impairment in abstinent MDMA ("Ecstasy") users. *Neurology*, **51,** 1532-1537.

BOWYER, J.F., DAVIES, D.L., SCHMUED, L., BROENING, H.W., NEWPORT, G.D., SLIKKER JR, W. & HOLSON, R.R. (1994). Further studies of the role of hyperthermia in methamphetamine neurotoxicity. *The Journal of Pharmacology and Experimental Therapeutics*, **268,** 1571-1580.

BRETT, R.R. & PRATT, J.A. (1990). Chronic handling modifies the anxiolytic effect of diazepam in the elevated plus-maze. *European Journal of Pharmacology*, **178,** 135-138.

BRILEY, M., CHOPIN, P. & MORET, C. (1990). Effect of serotonergic lesion on "anxious" behaviour measured in the elevated plus-maze test in the rat. *Psychopharmacology*, **101,** 187-189.

BROENING, H.W., BOWYER, J.F. & SLIKKER JR., W. (1995). Age-dependent sensitivity of rats to the long-term effects of the serotonergic neurotoxicant (±)-3,4-methylenedioxymethamphetamine (MDMA) correlates with the magnitude of the MDMA-induced thermal response. *The Journal of Pharmacology and Experimental Therapeutics*, **275,** 325-333.

BRONSTEIN, D.M. & HONG, J.-S. (1995). Effects of sulpiride and SCH 23390 on methamphetamine-induced changes in body temperature and lethality. *The Journal of Pharmacology and Experimental Therapeutics*, **274,** 943-950.

BROWN, C. & OSTERLOH, J. (1987). Multiple severe complications from recreational ingestion of MDMA ('Ecstasy'). *Journal of the American Medical Association*, **258,** 780-781.

BROWN, J.S., KALISH, H.I. & FARBER, I.E. (1951). Conditioned fear as revealed by magnitude of startle response to an auditory stimulus. *Journal of Experimental Psychology*, **41,** 317-328.

BRUNNER, H.G., NELEN, M., BREAKEFIELD, X.O., ROPERS, H.H. & VAN OOST, B.A. (1993). Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. *Science*, **262,** 578-80.

BUCHAN, A. & PULSINELLI, W.A. (1990). Hypothermia but not the *N*-methyl-D-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. *The Journal of Neuroscience*, **10,** 311-316.

BUSTO, R., DIETRICH, W.D., GLOBUS, M.Y., VALDÉS, I., SCHEINBERG, P. & GINSBERG, M.D. (1987). Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. *Journal of Cerebral Blood Flow and Metabolism*, **7,** 729-738.

CADET, J.L., LADENHEIM, B., BAUM, I., CARLSON, E. & EPSTEIN, C. (1994). CuZn-superoxide dismutase (CuZnSOD) transgenic mice show resistance to the lethal effects of methylenedioxyamphetamine (MDA) and of methylenedioxymethamphetamine (MDMA). *Brain Research*, **655**, 259-262.

CADET, J.L., LADENHEIM, B., HIRATA, H., ROTHMAN, R.B., ALI, S., CARLSON, E., EPSTEIN, C. & MORAN, T.H. (1995). Superoxide radicals mediate the biochemical effects of methylenedioxymethamphetamine (MDMA): evidence from using CuZn-superoxide dismutase transgenic mice. *Synapse*, **21**, 169-176.

CADET, J.-L., THIRIET, N. & JAYANTHI, S. (2001). Involvement of free radicals in MDMA-induced neurotoxicity in mice. *Annales de Médecine Interne*, **152**, 1S57-1S59.

CALLAHAN, B.T. & RICAURTE, G.A. (1998). Effect of 7-nitroindazole on body temperature and methamphetamine-induced dopamine toxicity. *NeuroReport*, **9**, 2691-2695.

CALLAHAN, B.T., CORD, B.J. & RICAURTE, G.A. (2001). Long-term impairment of anterograde axonal transport along fiber projections originating in the rostral raphe nuclei after treatment with fenfluramine or methylenedioxymethamphetamine. *Synapse*, **40**, 113-121.

CALLAWAY, C.W., WING, L.L. & GEYER, M.A. (1990). Serotonin release contributes to the locomotor stimulant effects of 3,4-methylenedioxymethamphetamine in rats. *The Journal of Pharmacology and Experimental Therapeutics*, **254**, 456-464.

CANCELA, L.M., BASSO, A.M., MARTIJENA, I.D., CAPRILES, N.R. & MOLINA, V.A. (2001). A dopaminergic mechanism is involved in the 'anxiogenic-like' response induced by chronic amphetamine treatment: a behavioral and neurochemical study. *Brain Research*, **909**, 179-186.

CERRUTI, C., SHENG, P., LADENHEIM, B., EPSTEIN, C.J. & CADET, J.-L. (1995). Involvement of oxidative and L-arginine-NO pathways in the neurotoxicity of drugs of abuse *in vitro*. *Clinical and Experimental Pharmacology and Physiology*, **22**, 381-382.

CHADWICK, I.S., LINSLEY, A., FREEMONT, A.J. & DORAN, B. (1991). Ecstasy, 3,4-methylenedioxymethamphetamine (MDMA), a fatality associated with coagulopathy and hyperthermia. *Journal of the Royal Society of Medicine*, **84**, 371.

CHANG, L., ERNST, T., GROB, C.S. & POLAND, R.E. (1999). Cerebral ^{1}H MRS alterations in recreational 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") users. *Journal of Magnetic Resonance Imaging*, **10**, 521-526.

CHAOULOFF, F., DURAND, M. & MORMÈDE, P. (1997). Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. *Behavioural Brain Research*, **85**, 27-35.

CHE, S., JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1995). Body temperature effect on methylenedioxyamphetamine - induced acute decrease in tryptophan hydroxylase activity. *European Journal of Pharmacology*, **293,** 447-453.

CHIUEH, C.C., KRISHNA, G., TULSI, P., OBATA, T., LANG, K., HUANG, S.-J. & MURPHY, D.L. (1992). Intracranial microdialysis of salicyclic acid to detect hydroxyl radical generation through dopamine auto-oxidation in the caudate nucleus: effects of MPP$^+$. *Free Radical Biology and Medicine*, **13,** 581-583.

CHIUEH, C.C., MIYAKE, H. & PENG, M.-T. (1993). Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced Parkinsonism. *Advances in Neurology*, **60,** 251-258.

CHRISTOPHERSEN, A.S. (2000). Amphetamine designer drugs - an overview and epidemiology. *Toxicology Letters*, **112-113,** 127-31.

COHEN, R.S. (1998). *The love drug. Marching to the beat of Ecstasy.* Binghamton: The Haworth Medical Press.

COLADO, M.I., MURRAY, T.K. & GREEN, A.R. (1993). 5-HT loss in rat brain following 3,4-methylenedioxymethamphetamine (MDMA), *p*-chloroamphetamine and fenfluramine administration and effects of chlormethiazole and dizocilpine. *British Journal of Pharmacology*, **108,** 583-589.

COLADO, M.I. & GREEN, A.R. (1994). A study of the mechanism of MDMA ('Ecstasy')-induced neurotoxicity of 5-HT neurones using chlormethiazole, dizocilpine and other protective compounds. *British Journal of Pharmacology*, **111,** 131-136.

COLADO, M.I. & GREEN, A.R. (1995). The spin trap reagent α-phenyl-*N*-tert-butyl nitrone prevents 'ecstasy'-induced neurodegeneration of 5-hydroxytryptamine neurones. *European Journal of Pharmacology*, **280,** 343-346.

COLADO, M.I., WILLIAMS, J.L. & GREEN, A.R. (1995). The hyperthermic and neurotoxic effects of 'Ecstasy' (MDMA) and 3,4 methylenedioxyamphetamine (MDA) in the Dark Agouti (DA) rat, a model of the CYP2D6 poor metabolizer phenotype. *British Journal Of Pharmacology*, **115,** 1281-1289.

COLADO, M.I., O'SHEA, E., GRANADOS, R., MURRAY, T.K. & GREEN, A.R. (1997a). *In vivo* evidence for free radical involvement in the degeneration of rat brain 5-HT following administration of MDMA ('ecstasy') and *p*-chloroamphetamine but not the degeneration following fenfluramine. *British Journal of Pharmacology*, **121,** 889-900.

COLADO, M.I., O'SHEA, E., GRANADOS, R., MISRA, A., MURRAY, T.K. & GREEN, A.R. (1997b). A study of the neurotoxic effect of MDMA ('ecstasy') on 5-HT neurones in the brains of mothers and neonates following administration of the drug during pregnancy. *British Journal of Pharmacology*, **121,** 827-833.

COLADO, M.I., GRANADOS, R., O'SHEA, E., ESTEBAN, B. & GREEN, A.R. (1998). Role of hyperthermia in the protective action of chlomethiazole against MDMA ('ecstasy')-induced neurodegeneration, comparison with the novel NMDA channel blocker AR-R15896AR. *British Journal of Pharmacology*, **124,** 479-484.

COLADO, M.I., O'SHEA, E., GRANADOS, R., ESTEBAN, B., MARTÍN, A.B. & GREEN, A.R. (1999a). Studies on the role of dopamine in the degeneration of 5-HT nerve endings in the brain of Dark Agouti rats following 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') administration. *British Journal of Pharmacology*, **126,** 911-924.

COLADO, M.I., O'SHEA, E., ESTEBAN, B., GRANADOS, R. & GREEN, A.R. (1999b). In vivo evidence against chlomethiazole being neuroprotective against MDMA ('ecstasy')-induced degeneration of rat brain 5-HT nerve terminals by a free radical scavenging mechanism. *Neuropharmacology*, **38,** 307-314.

COLADO, M.I., ESTEBAN, B., O'SHEA, E., MARTINEZ, I. & GREEN, A.R. (1999c). Effect of 7-nitroindazole on the hypothermia and the striatal dopamine loss induced by MDMA in mice. *British Journal of Pharmacology*, **127,** 70P.

COLADO, M.I., GRANADOS, R., O'SHEA, E., ESTEBAN, B. & GREEN, A.R. (1999d). The acute effect in rats of 3,4-methylenedioxyethamphetamine (MDEA, "Eve") on body temperature and long term degeneration of 5-HT neurones in brain: a comparison with MDMA ("Ecstasy"). *Pharmacology and Toxicology*, **84,** 261-266.

COLADO, M.I., CAMARERO, J., MECHAN, A.O., SANCHEZ, V., ESTEBAN, B., ELLIOTT, J.M. & GREEN, A.R. (2001). A study of the mechanisms involved in the neurotoxic action of 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') on dopamine neurones in mouse brain. *British Journal of Pharmacology* (submitted for publication).

COMMINS, D.L., VOSMER, G., VIRUS, R.M., WOOLVERTON, W.L., SCHUSTER, C.R. & SEIDEN, L.S. (1987). Biochemical and histological evidence that methylenedioxymethamphetamine (MDMA) is toxic to neurons in the rat brain. *The Journal of Pharmacology and Experimental Therapeutics*, **241,** 338-345.

COSTALL, B., JONES, B.J., KELLY, M.E., NAYLOR, R.J. & TOMKINS, D.M. (1989). Exploration of mice in a black and white test box: validation as a model of anxiety. *Pharmacology Biochemistry and Behavior*, **32,** 777-785.

CREGAN, E.F., PEELING, J., CORBETT, D., BUCHAN, A.M., SAUNDERS, J., AUER, R.N., GAO, M., MCCARTHY, D.J., EISMAN, M.S., CAMPBELL, T.M., MURRAY, R.J., STAGNITTO, M.L. & PALMER, G.C. (1997). [(*S*)-*alpha*-phenyl-2-pyridine-ethanamine dihydrochloride], a low affinity uncompetitive N-methyl-D-aspartic acid antagonist, is effective in rodent models of global and focal ischemia. *The Journal of Pharmacology and Experimental Therapeutics*, **283,** 1412-1424.

CREIGHTON, F.J., BLACK, D.L. & HYDE, C.E. (1991). 'Ecstasy' psychosis and flashbacks. *British Journal of Psychiatry*, **159,** 713-715.

CRESPI, D., MENNINI, T. & GOBBI, M. (1997). Carrier-dependent and Ca^{2+}-dependent 5-HT and dopamine release induced by (+)-amphetamine, 3,4-methylenedioxymethamphetamine, *p*-chloroamphetamine and (+)-fenfluramine. *British Journal of Pharmacology*, **121,** 1735-1743.

CROSS, A.J., JONES, J.A., BALDWIN, H.A. & GREEN, A.R. (1991). Neuroprotective activity of chlormethiazole following transient forebrain ischaemia in the gerbil. *British Journal of Pharmacology*, **104,** 406-411.

CRUZ, A.P.M., FREI, F. & GRAEFF, F.G. (1994). Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacology Biochemistry and Behavior*, **49,** 171-176.

DAFTERS, R.I. (1994). Effect of ambient temperature on hyperthermia and hyperkinesis induced by 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') in rats. *Psychopharmacology*, **114,** 505-508.

DAFTERS, R.I. (1995). Hyperthermia following MDMA administration in rats: effects of ambient temperature, water consumption, and chronic dosing. *Physiology and Behavior*, **58,** 877-882.

DAFTERS, R.I. & LYNCH, E. (1998). Persistent loss of thermoregulation in the rat induced by 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') but not by fenfluramine. *Psychopharmacology*, **138,** 207-212.

DAS, N.P. & RATTY, A.K. (1987). Studies on the effects of the narcotic alkaloids, cocaine, morphine, and codeine on nonenzymatic lipid peroxidation in rat brain mitochondria. *Biochemical Medicine and Metabolic Biology*, **37,** 258-264.

DAVISON, D. & PARROTT, A.C. (1997). Ecstasy (MDMA) in recreational users: self-reported psychological and physiological effects. *Human Psychopharmacology: Clinical and Experimental*, **12,** 221-226.

DAWSON, G.R. & TRICKLEBANK, M.D. (1995). Use of the elevated plus maze in the search for novel anxiolytic agents. *Trends in Pharmacological Sciences*, **16,** 33-36.

DE LA TORRE, R., FARRÉ, M., ORTUÑO, J., MAS, M., BRENNEISEN, R., ROSET, P.N., SEGURA, J. & CAMÍ, J. (2000). Non-linear pharmacokinetics of MDMA ('ecstasy') in humans. *British Journal of Clinical Pharmacology*, **49,** 104-109.

DE SOUZA, I., KELLY, J.P., HARKIN, A.J. & LEONARD, B.E. (1997). An appraisal of the pharmacological and toxicological effects of a single oral administration of 3,4-methylenedioxymethamphetamine (MDMA) in the rat. *Pharmacology and Toxicology*, **80,** 207-210.

DI MONTE, D.A., ROYLAND, J.E., JAKOWEC, M.W. & LANGSTROM, J.W. (1996). Role of nitric oxide in methamphetamine neurotoxicity: protection by 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase. *Journal of Neurochemistry*, **67,** 2443-2450.

DOWLING, G.P., MCDONOUGH III, E.T. & BOST, R.O. (1987). 'Eve' and 'Ecstasy'. A report of five deaths associated with the use of MDEA and MDMA. *Journal of the American Medical Association*, **257,** 1615-1617.

DUDLEY, M.W., WIECH, N.L., MILLER, F.P., CARR, A.A., CHENG, H.C., ROEBEL, L.E., DOHERTY, N.S., YAMAMURA, H.I., URSILLO, R.C. & PALFREYMAN, M.G. (1988). Pharmacological effects of MDL 11,939: a selective, centrally acting antagonist of 5-HT_2 receptors. *Drug Development Research*, **13,** 29-43.

EIKELBOOM, R. (1986). Learned anticipatory rise in body temperature due to handling. *Physiology and Behavior*, **37,** 649-653.

ESCORIHUELA, R.M., FERNÁNDEZ-TERUEL, A., GIL, L., AGUILAR, R., TOBEÑA, A. & DRISCOLL, P. (1999). Inbred Roman high- and low-avoidance rats: differences in anxiety, novelty-seeking, and shuttlebox behaviors. *Physiology and Behavior*, **67,** 19-26.

ESTEBAN, B., O'SHEA, E., CAMARERO, J., GREEN, A.R. & COLADO, M.I. (2001). 3,4-methylenedioxymethamphetamine induces monoamine release, but not toxicity, when administered centrally at a concentration occurring following a peripherally injected neurotoxic dose. *Psychopharmacology*, **154,** 251-260.

FARFEL, G.M. & SEIDEN, L.S. (1995a). Role of hypothermia in the mechanism of protection against serotonergic toxicity. I. Experiments using 3,4-methylenedioxymethamphetamine, dizocilpine, CGS 19755 and NBQX. *The Journal of Pharmacology and Experimental Therapeutics*, **272,** 860-867.

FARFEL, G.M. & SEIDEN, L.S. (1995b). Role of hypothermia in the mechanism of protection against serotonergic toxicity. II. Experiments with methamphetamine, *p*-chloroamphetamine, fenfluramine, dizocilpine and dextromethorphan. *The Journal of Pharmacology and Experimental Therapeutics*, **272,** 868-875.

FAUNT, J.E. & CROCKER, A.D. (1987). The effects of selective dopamine receptor agonists and antagonists on body temperature in rats. *European Journal of Pharmacology*, **133,** 243-247.

FERGER, B., TEISMANN, P., EARL, C.D., KUSCHINSKY, K. & OERTEL, W.H. (2000). The protective effects of PBN against MPTP toxicity are independent of hydroxyl radical trapping. *Pharmacology Biochemistry and Behavior*, **65,** 425-431.

FILE, S.E. & HYDE, J.R.G. (1977). The effects of *p*-chlorophenylalanine and ethanolamine-*O*-sulphate in an animal test of anxiety. *Journal of Pharmacy and Pharmacology*, **29,** 735-738.

FILE, S.E. & HYDE, J.R.G. (1978). Can social interaction be used to measure anxiety? *British Journal of Pharmacology*, **62,** 19-24.

FILE, S.E. (1980). The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. *Journal of Neuroscience Methods*, **2,** 219-238.

FILE, S.E. (1990). One-trial tolerance to the anxiolytic effects of chlordiazepoxide in the plus-maze. *Psychopharmacology*, **100,** 281-282.

FILE, S.E., MABBUTT, P.S. & HITCHCOTT, P.K. (1990). Characterisation of the phenomenon of "one-trial tolerance" to the anxiolytic effect of chlordiazepoxide in the elevated plus-maze. *Psychopharmacology*, **102,** 98-101.

FILE, S.E. (1992). Behavioural detection of anxiolytic action. In: *Experimental approaches to anxiety and depression*, pp. 25-44. Editors: Elliott, J.M., Heal, D.J. & Marsden, C.A. John Wiley & Sons Ltd.

FILE, S.E., ANDREWS, N., WU, P.Y., ZHARKOVSKY, Z. & ZANGROSSI, H. (1992). Modification of chlordiazepoxide's behavioural and neurochemical effects by handling and plus-maze experience. *European Journal of Pharmacology*, **218,** 9-14.

FILE, S.E., ZANGROSSI JR, H., VIANA, M. & GRAEFF, F.G. (1993a). Trial 2 in the elevated plus-maze: a different form of fear? *Psychopharmacology*, **111,** 491-494.

FILE, S.E., ZANGROSSI JR., H. & ANDREWS, N. (1993b). Social interaction and elevated plus-maze tests: changes in release and uptake of 5-HT and GABA. *Neuropharmacology*, **32,** 217-221.

FISCHER, C., HATZIDIMITRIOU, G., WLOS, J., KATZ, J. & RICAURTE, G. (1995). Reorganization of ascending 5-HT axon projections in animals previously exposed to the recreational drug (±)-3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy'). *The Journal of Neuroscience*, **15,** 5476-5485.

FLECKENSTEIN, A.E., HAUGHEY, H.M., METZGER, R.R., KOKOSHKA, J.M., RIDDLE, E.L., HANSON, J.E., GIBB, J.W. & HANSON, G.R. (1999). Differential effects of psychostimulants and related agents on dopaminergic and serotonergic transporter function. *European Journal of Pharmacology*, **382,** 45-49.

FLECKENSTEIN, A.E., GIBB, J.W. & HANSON, G.R. (2000). Differential effects of stimulants on monoaminergic transporters: Pharmacological consequences and implications for neurotoxicity. *European Journal of Pharmacology*, **406,** 1-13.

FULLER, R.W. (1985). Drugs altering serotonin synthesis and metabolism. In: *Neuropharmacology of serotonin*, pp. 1-20. Editor: Green, A.R. Oxford: Oxford University Press.

FURFINE, E.S., HARMON, M.F., PAITH, J.E., KNOWLES, R.G., SALTER, M., KIFF, R.J., DUFFY, C., HAZELWOOD, R., OPLINGER, J.A. & GARVEY, E.P. (1994). Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by *S*-methyl-*L*- thiocitrulline and *S*-ethyl-*L*-thiocitrulline. *The Journal of Biological Chemistry*, **269,** 26677-26683.

GARTHWAITE, J., GARTHWAITE, G., PALMER, R.M.J. & MONCADA, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *European Journal of Pharmacology*, **172,** 413-416.

GELLER, I., KULAK JR., J.T. & SEIFTER, J. (1962). The effects of chlordiazepoxide and chlorpromazine on a punishment discrimination. *Psychopharmacologia*, **3,** 374-385.

GERRA, G., ZAIMOVIC, A., FERRI, M., ZAMBELLI, U., TIMPANO, M., NERI, E., MARZOCCHI, G.F., DELSIGNORE, R. & BRAMBILLA, F. (2000). Long-lasting effects of (±)3,4-methylenedioxymethamphetamine (Ecstasy) on serotonin system function in humans. *Biological Psychiatry*, **47,** 127-136.

GIOVANNI, A., LIANG, L.P., HASTINGS, T.G. & ZIGMOND, M.J. (1995). Estimating hydroxyl radical content in rat brain using systemic and intraventricular salicylate: impact of methamphetamine. *Journal of Neurochemistry*, **64,** 1819-1825.

GOODWIN, G.M., DE SOUZA, R.J., GREEN, A.R. & HEAL, D.J. (1987). The pharmacology of the behavioural and hypothermic responses of rats to 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). *Psychopharmacology*, **91,** 506-511.

GORDON, C.J., WATKINSON, W.P., O'CALLAGHAN, J.P. & MILLER, D.B. (1991). Effects of 3,4-methylenedioxymethamphetamine on autonomic thermoregulatory responses of the rat. *Pharmacology Biochemistry and Behavior*, **38,** 339-344.

GOUGH, B., ALI, S.F., SLIKKER JR, W. & HOLSON, R.R. (1991). Acute effects of 3,4-methylenedioxymethamphetamine (MDMA) on monoamines in rat caudate. *Pharmacology Biochemistry and Behavior*, **39,** 619-623.

GRAEFF, F.G., GUIMARÃES, F.S., DE ANDRADE, T.G.C.S. & DEAKIN, J.F.W. (1996). Role of 5-HT in stress, anxiety, and depression. *Pharmacology Biochemistry and Behavior*, **54,** 129-141.

GRAHAM, D.G., TIFFANY, S.M., BELL JR, W.R. & GUTKNECHT, W.F. (1978). Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells *in vitro*. *Molecular Pharmacology*, **14,** 644-653.

GRAHAME-SMITH, D.G. (1971). Studies *in vivo* on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. *Journal of Neurochemistry*, **18,** 1053-1066.

GRANT, R.T. (1963). Vasodilatation and body warming in the rat. *Journal of Physiology*, **167,** 311-317.

GREEN, A.R., DE SOUZA, R.J., WILLIAMS, J.L., MURRAY, T.K. & CROSS, A.J. (1992). The neurotoxic effects of methamphetamine on 5-hydroxytryptamine and dopamine in brain: evidence for the protective effect of chlormethiazole. *Neuropharmacology*, **31,** 315-321.

GREEN, A.R., CROSS, A.J. & GOODWIN, G.M. (1995). Review of the pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy"). *Psychopharmacology*, **119,** 247-260.

GREEN, A.R. (1998). Clomethiazole (Zendra®) in acute ischemic stroke: basic pharmacology and biochemistry and clinical efficacy. *Pharmacology and Therapeutics*, **80,** 123-147.

GREER, G. & STRASSMAN, R.J. (1985). Information on "Ecstasy". *American Journal of Psychiatry*, **142,** 1391.

GRIEBEL, G., PERRAULT, G. & SANGER, D.J. (1997a). A comparative study of the effects of selective and non-selective 5-HT_2 receptor subtype antagonists in rat and mouse models of anxiety. *Neuropharmacology*, **36,** 793-802.

GRIEBEL, G., RODGERS, R.J., PERRAULT, G. & SANGER, D.J. (1997b). Risk assessment behaviour: evaluation of utility in the study of 5-HT-related drugs in the rat elevated plus-maze test. *Pharmacology Biochemistry and Behavior*, **57,** 817-827.

GRINSPOON, L. & BAKALAR, J.B. (1986). Can drugs be used to enhance the psychotherapeutic process? *American Journal of Psychotherapy*, **40,** 393-404.

GUDELSKY, G.A., YAMAMOTO, B.K. & NASH, J.F. (1994). Potentiation of 3,4-methylenedioxymethamphetamine-induced dopamine release and serotonin neurotoxicity by 5-HT2 receptor agonists. *European Journal of Pharmacology*, **264,** 325-330.

GUDELSKY, G.A. (1996). Effect of ascorbate and cysteine on the 3,4-methylenedioxymethamphetamine-induced depletion of brain serotonin. *Journal of Neural Transmission*, **103,** 1397-1404.

GUDELSKY, G.A. & NASH, J.F. (1996). Carrier-mediated release of serotonin by 3,4-methylenedioxymethamphetamine: implications for serotonin-dopamine interactions. *Journal of Neurochemistry*, **66,** 243-249.

HALL, C.S. (1934a). Drive and emotionality: factors associated with adjustment in the rat. *Journal of Comparative Psychology*, **17,** 89-108.

HALL, C.S. (1934b). Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. *Journal of Comparative Psychology*, **18,** 385-404.

HALL, C.S. (1936). Emotional behavior in the rat. III. The relationship between emotionality and ambulatory activity. *Journal of Comparative Psychology*, **22,** 345-352.

HALL, F.S., DE VRIES, A.C., FONG, G.W., HUANG, S. & PERT, A. (1999). Effects of 5,7-dihydroxytryptamine depletion of tissue serotonin levels on extracellular serotonin in the striatum assessed with in vivo microdialysis: relationship to behavior. *Synapse*, **33,** 16-25.

HALLIWELL, B. & KAUR, H. (1997). Hydroxylation of salicylate and phenylalanine as assays for hydroxyl radicals: a cautionary note visited for the third time. *Free Radical Research*, **27,** 239-244.

HANDLEY, S.L. & MITHANI, S. (1984). Effects of alpha-adrenoceptor agonists and antagonists in a maze exploration model of 'fear'-motivated behaviour. *Naunyn Schmiedeberg's Archives of Pharmacology*, **327,** 1-5.

HANDLEY, S.L. & MCBLANE, J.W. (1993a). An assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs. *Journal of Pharmacological and Toxicological Methods*, **29,** 129-138.

HANDLEY, S.L. & MCBLANE, J.W. (1993b). 5HT drugs in animal models of anxiety. *Psychopharmacology*, **112,** 13-20.

HANDLEY, S.L., MCBLANE, J.W., CRITCHLEY, M.A.E. & NJUNG'E, K. (1993). Multiple serotonin mechanisms in animal models of anxiety: environmental, emotional and cognitive factors. *Behavioural Brain Research*, **38,** 203-210.

HATZIDIMITRIOU, G., MCCANN, U.D. & RICAURTE, G.A. (1999). Altered serotonin innervation patterns in the forebrain of monkeys treated with (±)3,4-methylenedioxymethamphetamine seven years previously: factors influencing abnormal recovery. *The Journal of Neuroscience*, **19,** 5096-5107.

HEGADOREN, K.M., BAKER, G.B. & BOURIN, M. (1999). 3,4-methylenedioxy analogues of amphetamine: defining the risks to humans. *Neuroscience and Biobehavioral Reviews*, **23,** 539-553.

HEISLER, L.K., CHU, H-M., BRENNAN, T.J., DANAO, J.A., BAJWA, P., PARSONS, L.H. & TECOTT, L.H. (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT_{1A} receptor mutant mice. *Proceedings of the National Academy of Sciences of the United States of America*, **95,** 15049-15054.

HENRY, J.A. (1992). Ecstasy and the dance of death. *British Medical Journal*, **305,** 5-6.

HENRY, J.A., JEFFREYS, K.J. & DAWLING, S. (1992). Toxicity and deaths from 3,4-methylenedioxymethamphetamine ("ecstasy"). *The Lancet*, **340,** 384-387.

HEWITT, K.E. & GREEN, A.R. (1994). Chlormethiazole, dizocilpine and haloperidol prevent the degeneration of serotonergic nerve terminals induced by administration of MDMA ('Ecstasy') to rats. *Neuropharmacology*, **33,** 1589-1595.

HIRAMATSU, M., KUMAGAI, Y., UNGER, S.E. & CHO, A.K. (1990). Metabolism of methylenedioxymethamphetamine: formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. *The Journal of Pharmacology and Experimental Therapeutics*, **254,** 521-527.

HOGG, S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacology Biochemistry and Behavior*, **54,** 21-30.

HOLMES, A. & RODGERS, R.J. (1998). Responses of Swiss–Webster mice to repeated plus-maze experience: further evidence for a qualitative shift in emotional state? *Pharmacology Biochemistry and Behavior*, **60,** 473-488.

HOLMES, A. & RODGERS, R.J. (1999). Influence of spatial and temporal manipulations on the anxiolytic efficacy of chlordiazepoxide in mice previously exposed to the elevated plus-maze. *Neuroscience and Biobehavioral Reviews*, **23,** 971-980.

HOLMES, A. (2001). Targeted gene mutation approaches to the study of anxiety-like behavior in mice. *Neuroscience and Biobehavioral Reviews*, **25,** 261-273.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). VII. International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacological Reviews*, **46,** 157-203.

HUANG, Z., HUANG, P.L., PANAHIAN, N., DALKARA, T., FISHMAN, M.C. & MOSKOWITZ, M.A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science*, **265,** 1883-1885.

IMAM, S.Z., CROW, J.P., NEWPORT, G.D., ISLAM, F., SLIKKER JR, W. & ALI, S.F. (1999). Methamphetamine generates peroxynitrite and produces dopaminergic neurotoxicity in mice: protective effects of peroxynitrite decomposition catalyst. *Brain Research*, **837,** 15-21.

IMAM, S.Z. & ALI, S.F. (2000). Selenium, an antioxidant, attenuates methamphetamine-induced dopaminergic toxicity and peroxynitrite generation. *Brain Research*, **855,** 186-191.

IMAM, S.Z., NEWPORT, G.D., ITZHAK, Y., CADET, J.L., ISLAM, F., SLIKKER JR, W. & ALI, S.F. (2001). Peroxynitrite plays a role in methamphetamine-induced dopaminergic neurotoxicity: evidence from mice lacking neuronal nitric oxide synthase gene or overexpressing copper-zinc superoxide dismutase. *Journal of Neurochemistry*, **76,** 745-749.

INSEL, T.R., BATTAGLIA, G., JOHANNESSEN, J.N., MARRA, S. & DE SOUZA, E.B. (1989). 3,4-methylenedioxymethamphetamine ("Ecstasy") selectively destroys brain serotonin terminals in Rhesus monkeys. *The Journal of Pharmacology and Experimental Therapeutics*, **249,** 713-720.

ITZHAK, Y. & ALI, S.F. (1996). The neuronal nitric oxide synthase inhibitor, 7-nitroindazole, protects against methamphetamine-induced neurotoxicity in vivo. *Journal of Neurochemistry*, **67,** 1770-1773.

ITZHAK, Y., GANDIA, C., HUANG, P.L. & ALI, S.F. (1998). Resistance of neuronal nitric oxide synthase-deficient mice to methamphetamine-induced dopaminergic neurotoxicity. *The Journal of Pharmacology and Experimental Therapeutics*, **284,** 1040-1047.

ITZHAK, Y., MARTIN, J.L. & ALI, S.F. (2000). nNOS inhibitors attenuate methamphetamine-induced dopaminergic neurotoxicity but not hyperthermia in mice. *NeuroReport*, **11,** 2943-2946.

IYER, R.N. & BRADBERRY, C.W. (1996). Serotonin-mediated increase in prefrontal cortex dopamine release: pharmacological characterization. *The Journal of Pharmacology and Experimental Therapeutics*, **277**, 40-47.

JAYANTHI, S., LADENHEIM, B., ANDREWS, A.M. & CADET, J.L. (1999). Overexpression of human copper/zinc superoxide dismutase in transgenic mice attenuates oxidative stress caused by methylenedioxymethamphetamine (Ecstasy). *Neuroscience*, **91**, 1379-1387.

JOHNSON, E.A., SHARP, D.S. & MILLER, D.B. (2000). Restraint as a stressor in mice: against the dopaminergic neurotoxicity of D-MDMA, low body weight mitigates restraint-induced hypothermia and consequent neuroprotection. *Brain Research*, **875**, 107-118.

JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1989). Effect of MK-801 on the decrease in tryptophan hydroxylase induced by methamphetamine and its methylenedioxy analog. *European Journal of Pharmacology*, **165**, 315-318.

JOHNSON, M., MITROS, K., STONE, D.M., ZOBRIST, R., HANSON, G.R. & GIBB, J.W. (1992). Effect of flunarizine and nimodipine on the decrease in tryptophan hydroxylase activity induced by methamphetamine and 3,4-methylenedioxymethamphetamine. *The Journal of Pharmacology and Experimental Therapeutics*, **261**, 586-591.

JOHNSON, M.P., HOFFMAN, A.J. & NICHOLS, D.E. (1986). Effects of the enantiomers of MDA, MDMA and related analogues on [^{3}H]serotonin and [^{3}H]dopamine release from superfused rat brain slices. *European Journal of Pharmacology*, **132**, 269-276.

KEHNE, J.H., KETTELER, H.J., MCCLOSKEY, T.C., SULLIVAN, C.K., DUDLEY, M.W. & SCHMIDT, C.J. (1996a). Effects of the selective 5-HT$_{2A}$ receptor antagonist MDL 100,907 on MDMA-induced locomotor stimulation in rats. *Neuropsychopharmacology*, **15**, 116-124.

KEHNE, J.H., BARON, B.M., CARR, A.A., CHANEY, S.F., ELANDS, J., FELDMAN, D.J., FRANK, R.A., VAN GIERSBERGEN, P.L., MCCLOSKEY, T.C., JOHNSON, M.P., MCCARTY, D.R., POIROT, M., SENYAH, Y., SIEGEL, B.W. & WIDMAIER, C. (1996b). Preclinical characterization of the potential of the putative atypical antipsychotic MDL 100,907 as a potent 5-HT$_{2A}$ antagonist with a favorable CNS safety profile. *The Journal of Pharmacology and Experimental Therapeutics*, **277**, 968-981.

KENNETT, G.A., WOOD, M.D., BRIGHT, F., TRAIL, B., RILEY, G., HOLLAND, V., AVENELL, K.Y., STEAN, T., UPTON, N., BROMIDGE, S., FORBES, I.T., BROWN, A.M., MIDDLEMISS, D.N. & BLACKBURN, T.P. (1997). SB 242084, a selective and brain penetrant 5-HT$_{2C}$ receptor antagonist. *Neuropharmacology*, **36**, 609-620.

KENNETT, G.A., TRAIL, B. & BRIGHT, F. (1998). Anxiolytic-like actions of BW 723C86 in the rat Vogel conflict test are 5-HT$_{2B}$ receptor mediated. *Neuropharmacology*, **37**, 1603-1610.

KENNETT, G., LIGHTOWLER, S., TRAIL, B., BRIGHT, F. & BROMIDGE, S. (2000). Effects of RO 60 0175, a 5-HT_{2C} receptor agonist, in three animal models of anxiety. *European Journal of Pharmacology*, **387,** 197-204.

KING, S.M. (1999). Escape-related behaviours in an unstable elevated and exposed environment. I. A new behavioural model of extreme anxiety. *Behavioural Brain Research*, **98,** 113-126.

KISH, S.J., FURUKAWA, Y., ANG, L., VORCE, S.P. & KALASINSKY, K.S. (2000). Striatal serotonin is depleted in brain of a human MDMA (Ecstasy) user. *Neurology*, **55,** 294-296.

KITA, T., MATSUNARI, Y., SARAYA, T., SHIMADA, K., O'HARA, K., KUBO, K., WAGNER, G.C. & NAKASHIMA, T. (2000). Evaluation of the effects of α-phenyl-N-*tert*-butyl nitrone pretreatment on the neurobehavioral effects of methamphetamine. *Life Sciences*, **67,** 1559-1571.

KOCH, S. & GALLOWAY, M.P. (1997). MDMA induced dopamine release in vivo: role of endogenous serotonin. *Journal of Neural Transmission*, **104,** 135-146.

KOE, B.K. & WEISSMAN, A. (1968). The pharmacology of para-chlorophenylalanine, a selective depletor of serotonin stores. *Advances in Pharmacology*, **6,** Supplement 1, 29-47.

KÖHLER, C. & LORENS, S.A. (1978). Open field activity and avoidance behavior following serotonin depletion: a comparison of the effects of parachlorophenylalanine and electrolytic midbrain raphe lesions. *Pharmacology Biochemistry and Behavior*, **8,** 223-233.

KRUK, Z.L. & PYCOCK, C.J. (1991). *Neurotransmitters and drugs*. Third Edition. London: Chapman & Hall.

LAVERTY, R. & LOGAN, B.J. (1990). Protection by MK 801 and other drugs of methylenedioxymethamphetamine (MDMA) neurotoxicity in rats and mice. *European Journal of Pharmacology*, **183,** 451-452.

LEW, R., SABOL, K.E., CHOU, C., VOSMER, G.L., RICHARDS, J. & SEIDEN, L.S. (1996). Methylenedioxymethamphetamine-induced serotonin deficits are followed by partial recovery over a 52-week period. Part II: radioligand binding and autoradiography studies. *The Journal of Pharmacology and Experimental Therapeutics*, **276,** 855-865.

LIECHTI, M.E. & VOLLENWEIDER, F.X. (2000). Acute psychological and physiological effects of MDMA ("Ecstasy") after haloperidol pretreatment in healthy humans. *European Neuropsychopharmacology*, **10,** 289-295.

LIM, H.K. & FOLTZ, R.L. (1988). In vivo and in vitro metabolism of 3,4-(methylenedioxy)methamphetamine in the rat: identification of metabolites using an ion trap detector. *Chemical Research in Toxicology*, **1,** 370-378.

LIM, H.K. & FOLTZ, R.L. (1991a). *In vivo* formation of aromatic hydroxylated metabolites of 3,4-(methylenedioxy)methamphetamine in the rat: identification by ion trap tandem mass spectrometric (MS/MS and MS/MS/MS) techniques. *Biological Mass Spectrometry*, **20,** 677-686.

LIM, H.K. & FOLTZ, R.L. (1991b). Ion trap tandem mass spectrometric evidence for the metabolism of 3,4-(methylenedioxy)methamphetamine to the potent neurotoxins 2,4,5-trihydroxymethamphetamine and 2,4,5-trihydroxyamphetamine. *Chemical Research in Toxicology*, **4,** 626-632.

LIN, H.Q., BURDEN, P.M., CHRISTIE, M.J. & JOHNSTON, G.A.R. (1999). The anxiogenic-like and anxiolytic-like effects of MDMA on mice in the elevated plus-maze: A comparison with amphetamine. *Pharmacology Biochemistry and Behavior*, **62,** 403-408.

LISTER, R.G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology*, **92,** 180-185.

LOGAN, B.J., LAVERTY, R., SANDERSON, W.D. & YEE, Y.B. (1988). Differences between rats and mice in MDMA (methylenedioxymethamphetamine) neurotoxicity. *European Journal of Pharmacology*, **152,** 227-234.

LUCKI, I. (1998). The spectrum of behaviors influenced by serotonin. *Biological Psychiatry*, **44,** 151-162.

MALBERG, J.E., SABOL, K.E. & SEIDEN, L.S. (1996). Co-administration of MDMA with drugs that protect against MDMA neurotoxicity produces different effects on body temperature in the rat. *The Journal of Pharmacology and Experimental Therapeutics*, **278,** 258-267.

MALBERG, J.E. & SEIDEN, L.S. (1998). Small changes in ambient temperature cause large changes in 3,4-methylenedioxymethamphetamine (MDMA)-induced serotonin neurotoxicity and core body temperature in the rat. *The Journal of Neuroscience*, **18,** 5086-5094.

MALDONADO, E. & NAVARRO, J.F. (2001). MDMA ('ecstasy') exhibits an anxiogenic-like activity in social encounters between male mice. *Pharmacological Research*, **44,** 27-31.

MALLICK, A. & BODENHAM, A.R. (1997). MDMA induced hyperthermia: a survivor with an initial body temperature of 42.9 °C. *Journal of Accident and Emergency Medicine*, **14,** 336-338.

MAREK, G.J., VOSMER, G. & SEIDEN, L.S. (1990). Dopamine uptake inhibitors block long-term neurotoxic effects of methamphetamine upon dopaminergic neurons. *Brain Research*, **513,** 274-279.

MARSTON, H.M., REID, M.E., LAWRENCE, J.A., OLVERMAN, H.J. & BUTCHER, S.P. (1999). Behavioural analysis of the acute and chronic effects of MDMA treatment in the rat. *Psychopharmacology*, **144,** 67-76.

MARTÍN, F. & ARTIGAS, F. (1992). Simultaneous effects of *p*-chloroamphetamine, *d*-fenfluramine, and reserpine on free and stored 5-hydroxytryptamine in brain and blood. *Journal of Neurochemistry*, **59,** 1138-1144.

MAZZOLA-POMIETTO, P., AULAKH, C.S., WOZNIAK, K.M., HILL, J.L. & MURPHY, D.L. (1995). Evidence that 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-induced hyperthermia in rats is mediated by stimulation of 5-HT_{2A} receptors. *Psychopharmacology*, **117,** 193-199.

MAZZOLA-POMIETTO, P., AULAKH, C.S., WOZNIAK, K.M. & MURPHY, D.L. (1996). Evidence that *m*-chlorophenylpiperazine-induced hyperthermia in rats is mediated by stimulation of 5-HT_{2C} receptors. *Psychopharmacology*, **123,** 333-339.

MCCALL, R.B. & SCHUETTE, M.R. (1984). Evidence for an *alpha*-1 receptor-mediated central sympathoinhibitory action of ketanserin. *The Journal of Pharmacology and Experimental Therapeutics*, **228,** 704-710.

MCCANN, U.D. & RICAURTE, G.A. (1991). Lasting neuropsychiatric sequelae of (±)methylenedioxymethamphetamine ('Ecstasy') in recreational users. *Journal of Clinical Psychopharmacology*, **11,** 302-305.

MCCANN, U.D. & RICAURTE, G.A. (1992). MDMA ("Ecstasy") and panic disorder: induction by a single dose. *Biological Psychiatry*, **32,** 950-953.

MCCANN, U.D., RIDENOUR, A., SHAHAM, Y. & RICAURTE, G.A. (1994). Serotonin neurotoxicity after (±)3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy'): a controlled study in humans. *Neuropsychopharmacology*, **10,** 129-138.

MCCANN, U.D., SLATE, S.O. & RICAURTE, G.A. (1996). Adverse reactions with 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy'). *Drug Safety*, **15,** 107-115.

MCCANN, U.D., SZABO, Z., SCHEFFEL, U., DANNALS, R.F. & RICAURTE, G.A. (1998). Positron emission tomographic evidence of toxic effect of MDMA ('Ecstasy') on brain serotonin neurons in human beings. *The Lancet*, **352,** 1433-1437.

MCCANN, U.D., ELIGULASHVILI, V., MERTL, M., MURPHY, D.L. & RICAURTE, G.A. (1999). Altered neuroendocrine and behavioral responses to *m*-chlorophenylpiperazine in 3,4-methylenedioxymethamphetamine (MDMA) users. *Psychopharmacology*, **147,** 56-65.

MCCANN, U.D. & RICAURTE, G.A. (2001). Caveat emptor: Editors beware. *Neuropsychopharmacology*, **24,** 333-334.

MCCREARY, A.C., BANKSON, M.G. & CUNNINGHAM, K.A. (1999). Pharmacological studies of the acute and chronic effects of (+)-3,4-methylenedioxymethamphetamine on locomotor activity: role of 5-hydroxytryptamine$_{1A}$ and 5-hydroxytryptamine$_{1B/1D}$ receptors. *The Journal of Pharmacology and Experimental Therapeutics*, **290,** 965-973.

MCGUIRE, P. & FAHY, T. (1991). Chronic paranoid psychosis after misuse of MDMA ("ecstasy"). *British Medical Journal*, **302,** 697.

MCGUIRE, P.K., COPE, H. & FAHY, T.A. (1994). Diversity of psychopathology associated with use of 3,4-methylenedioxymethamphetamine ('Ecstasy'). *British Journal of Psychiatry*, **165,** 391-395.

MCGUIRE, P. (2000). Long term psychiatric and cognitive effects of MDMA use. *Toxicology Letters*, **112-113,** 153-156.

MCKENNA, D.J. & PEROUTKA, S.J. (1990). Neurochemistry and neurotoxicity of 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy'). *Journal of Neurochemistry*, **54,** 14-22.

MCNAMARA, M.G., KELLY, J.P. & LEONARD, B.E. (1995). Some behavioural and neurochemical aspects of subacute (±)3,4-methylenedioxymethamphetamine administration in rats. *Pharmacology Biochemistry and Behavior*, **52,** 479-484.

MECHAN, A.O., COLADO, M.I., ESTEBAN, B., ELLIOTT, J.M. & GREEN, A.R. (2000). Evidence against MDMA-induced hyperthermia being mediated by 5-HT release in the brain. *British Journal of Pharmacology*, **131,** 153P.

MECHAN, A.O., O'SHEA, E., ELLIOTT, J.M., COLADO, M.I. & GREEN, A.R. (2001a). A neurotoxic dose of 3,4-methylenedioxymethamphetamine (MDMA; ecstasy) to rats results in a long term defect in thermoregulation. *Psychopharmacology*, **155,** 413-418.

MECHAN, A.O., ESTEBAN, B., O'SHEA, E., ELLIOTT, J.M., COLADO, M.I. & GREEN, A.R. (2001b). The pharmacology of the acute hyperthermic response that follows administration of 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') to rats. *British Journal of Pharmacology*, (in press).

METZGER, R.R., HAUGHEY, H.M., WILKINS, D.G., GIBB, J.W., HANSON, G.R. & FLECKENSTEIN, A.E. (2000). Methamphetamine-induced rapid decrease in dopamine transporter function: role of dopamine and hyperthermia. *The Journal of Pharmacology and Experimental Therapeutics*, **295,** 1077-1085.

MILLER, D.B. & O'CALLAGHAN, J.P. (1993). The interactions of MK-801 with the amphetamine analogues D-methamphetamine (D-METH), 3,4-methylenedioxymethamphetamine (D-MDMA) or D-fenfluramine (D-FEN): neural damage and neural protection. *Annals of the New York Academy of Sciences*, **679,** 321-324.

MILLER, D.B. & O'CALLAGHAN, J.P. (1994). Environment-, drug- and stress-induced alterations in body temperature affect the neurotoxicity of substituted amphetamines in the C57BL/6J mouse. *The Journal of Pharmacology and Experimental Therapeutics*, **270,** 752-760.

MILLER, D.B. & O'CALLAGHAN, J.P. (1995). The role of temperature, stress, and other factors in the neurotoxicity of the substituted amphetamines 3,4-methylenedioxymethamphetamine and fenfluramine. *Molecular Neurobiology*, **11,** 177-192.

MILROY, C.M., CLARK, J.C. & FORREST, A.R.W. (1996). Pathology of deaths associated with ecstasy and 'Eve' misuse. *Journal of Clinical Pathology*, **49,** 149-153.

MILTON, A.S. (1977). The hypothalamus and the pharmacology of thermoregulation. In: *Pharmacology of the hypothalamus*. Editors: Cox, B., Morris, I.D. & Weston, A.H. London: The Macmillan Press Ltd.

MOHAGHEGH, R.A., SOULSBY, M.E., SKINNER, R.D. & KENNEDY, R.H. (1997). The interaction between the central and peripheral nervous systems in mediating the thermic effect of methamphetamine. *Annals of the New York Academy of Sciences*, **813,** 197-203.

MOLLIVER, M.E., BERGER, U.V., MAMOUNAS, L.A., MOLLIVER, D.C., O'HEARN, E. & WILSON, M.A. (1990). Neurotoxicity of MDMA and related compounds: anatomic studies. *Annals of the New York Academy of Sciences*, **600,** 640-661.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*, **43,** 109-142.

MONTGOMERY, K.C. (1952). Exploratory behavior and its relation to spontaneous alternation in a series of maze exposures. *Journal of Comparative and Physiological Psychology*, **45,** 50-57.

MONTGOMERY, K.C. (1955). The relation between fear induced by novel stimulation and exploratory behaviour. *Journal of Comparative and Physiological Psychology*, **48,** 254-260.

MONTGOMERY, K.C. & MONKMAN, J.A. (1955). The relation between fear and exploratory behavior. *Journal of Comparative and Physiological Psychology*, **48,** 132-136.

MORDENTI, J. (1986). Man versus beast: pharmacokinetic scaling in mammals. *Journal of Pharmaceutical Sciences*, **75,** 1028-1040.

MORGAN, M.J. (1998). Recreational use of "Ecstasy" (MDMA) is associated with elevated impulsivity. *Neuropsychopharmacology*, **19,** 252-264.

MORGAN, M.J. (1999). Memory deficits associated with recreational use of "ecstasy" (MDMA). *Psychopharmacology*, **141,** 30-36.

MORGAN, M.J. (2000). Ecstasy (MDMA): a review of its possible persistent psychological effects. *Psychopharmacology*, **152,** 230-248.

MORLEY, K.C. & MCGREGOR, I.S. (2000). (±)-3,4-Methylenedioxymethamphetamine (MDMA, 'Ecstasy') increases social interaction in rats. *European Journal of Pharmacology*, **408,** 41-49.

MURRAY, T.K., WILLIAMS, J.L., MISRA, A., COLADO, M.I. & GREEN, A.R. (1996). The spin trap reagent PBN attenuates degeneration of 5-HT neurones in rat brain induced by *p*-chloroamphetamine but not fenfluramine. *Neuropharmacology*, **35,** 1615-1620.

MYERS, R.D. (1981). Serotonin and thermoregulation: old and new views. *Journal of Physiology*, **77,** 505-513.

NARAYAN, M., BERLINER, L.J., MEROLA, A.J., DIAZ, P.T. & CLANTON, T.L. (1997). Biological reactions of peroxynitrite: evidence for an alternative pathway of salicylate hydroxylation. *Free Radical Research*, **27,** 63-72.

NARAYANAN, K. & GRIFFITH, O.W. (1994). Synthesis of L-thiocitrulline, L-homothiocitrulline, and S-methyl-L-thiocitrulline: a new class of potent nitric oxide synthase inhibitors. *Journal of Medicinal Chemistry*, **37,** 885-887.

NASH JR., J.F., MELTZER, H.Y. & GUDELSKY, G.A. (1988). Elevation of serum prolactin and corticosterone concentrations in the rat after the administration of 3,4-methylenedioxymethamphetamine. *The Journal of Pharmacology and Experimental Therapeutics*, **245,** 873-879.

NASH, J.F. (1990). Ketanserin pretreatment attenuates MDMA-induced dopamine release in the striatum as measured by *in vivo* microdialysis. *Life Sciences*, **47,** 2401-2408.

NASH, J.F. & BRODKIN, J. (1991). Microdialysis studies on 3,4-methylenedioxymethamphetamine-induced dopamine release: effect of dopamine uptake inhibitors. *The Journal of Pharmacology and Experimental Therapeutics*, **259,** 820-825.

NASH, J.F. & YAMAMOTO, B.K. (1992). Methamphetamine neurotoxicity and striatal glutamate release: comparison to 3,4-methylenedioxymethamphetamine. *Brain Research*, **581,** 237-243.

NISIJIMA, D., YOSHINO, T., YUI, K. & KATOH, S. (2001). Potent serotonin $(5\text{-HT})_{2A}$ receptor antagonists completely prevent the development of hyperthermia in an animal model of the 5-HT syndrome. *Brain Research*, **890,** 23-31.

NIXDORF, W.I., BURROWS, K.B., GUDELSKY, G.A. & YAMAMOTO, B.K. (2001). Enhancement of 3,4-methylenedioxymethamphetamine neurotoxicity by the energy inhibitor malonate. *Journal of Neurochemistry*, **77,** 647-654.

OBRADOVIC, T., IMEL, K.M. & WHITE, S.R. (1998). Repeated exposure to methylenedioxymethamphetamine (MDMA) alters nucleus accumbens neuronal responses to dopamine and serotonin. *Brain Research*, **785,** 1-9.

O'CALLAGHAN, J.P. & MILLER, D.B. (1994). Neurotoxicity profiles of substituted amphetamines in the C57BL/6J mouse. *The Journal of Pharmacology and Experimental Therapeutics*, **270,** 741-751.

O'HEARN, E., BATTAGLIA, G., DE SOUZA, E.B., KUHAR, M.J. & MOLLIVER, M.E. (1988). Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain: immunocytochemical evidence for neurotoxicity. *The Journal of Neuroscience*, **8,** 2788-2803.

OLAUSSON, P., ENGEL, J.A. & SÖDERPALM, B. (2000). Effects of serotonergic manipulations on the behavioral sensitization and disinhibition associated with repeated amphetamine treatment. *Pharmacology Biochemistry and Behavior*, **66,** 211-220.

O'LOINSIGH, E.D., BOLAND, G., KELLY, J.P. & O'BOYLE, K.M. (2001). Behavioural, hyperthermic and neurotoxic effects of 3,4-methylenedioxymethamphetamine analogues in the Wistar rat. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **25,** 621-638.

O'NEILL, M.J., MURRAY, T.K., MCCARTY, D.R., HICKS, C.A., DELL, C.P., PATRICK, K.E., WARD, M.A., OSBORNE, D.J., WIERNICKI, T.R., ROMAN, C.R., LODGE, D., FLEISCH, J.H. & SINGH, J. (2000). ARL 17477, a selective nitric oxide synthase inhibitor, with neuroprotective effects in animal models of global and focal cerebral ischaemia. *Brain Research*, **871,** 234-244.

O'SHEA, E., GRANADOS, R., ESTEBAN, B., COLADO, M.I. & GREEN, A.R. (1998). The relationship between the degree of neurodegeneration of rat brain 5-HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy'). *Neuropharmacology*, **37,** 919-926.

O'SHEA, E., ESTEBAN, B., CAMARERO, J., GREEN, A.R. & COLADO, M.I. (2001). Effect of GBR 12909 and fluoxetine on the acute and long term changes induced by MDMA ('ecstasy') on the 5-HT and dopamine concentrations in mouse brain. *Neuropharmacology*, **40,** 65-74.

PALANZA, P. (2001). Animal models of anxiety and depression: how are females different? *Neuroscience and Biobehavioral Reviews*, **25,** 219-233.

PARIS, J.M. & CUNNINGHAM, K.A. (1991). Lack of serotonin neurotoxicity after intraraphe microinjection of (+)-3,4-methylenedioxymethamphetamine (MDMA). *Brain Research Bulletin*, **28,** 115-119.

PARROTT, A.C. & STUART, M. (1997). Ecstasy (MDMA), amphetamine, and LSD: comparative mood profiles in recreational polydrug users. *Human Psychopharmacology*, **12,** 501-504.

PARROTT, A.C. & LASKY, J. (1998). Ecstasy (MDMA) effects upon mood and cognition: before, during and after a Saturday night dance. *Psychopharmacology*, **139,** 261-268.

PARROTT, A.C., SISK, E. & TURNER, J.J.D. (2000). Psychobiological problems in heavy 'ecstasy' (MDMA) polydrug users. *Drug and Alcohol Dependence*, **60,** 105-110.

PAWLOWSKI, L. (1981). Different action of 5-hydroxytryptamine (5-HT) uptake inhibitors on fenfluramine - but not *p*-chloroamphetamine-induced hyperthermia in rats. *Journal of Pharmacy and Pharmacology*, **33,** 538-540.

PEHEK, E.A., MCFARLANE, H.G., MAGUSCHAK, K., PRICE, B. & PLUTO, C.P. (2001). M100,907, a selective 5-HT_{2A} antagonist, attenuates dopamine release in the rat medial prefrontal cortex. *Brain Research*, **888,** 51-59.

PELLOW, S., CHOPIN, P., FILE, S.E. & BRILEY, M. (1985). Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*, **14,** 149-167.

PELLOW, S. & FILE, S.E. (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacology Biochemistry and Behavior*, **24,** 525-529.

PEROUTKA, S.J. (1987). Incidence of recreational use of 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") on an undergraduate campus. *The New England Journal of Medicine*, **317,** 1542-1543.

PEROUTKA, S.J., NEWMAN, H. & HARRIS, H. (1988). Subjective effects of 3,4-methylenedioxymethamphetamine in recreational users. *Neuropsychopharmacology*, **1,** 273-277.

PERRON, R.R., TYSON, R.L. & SUTHERLAND, G.R. (2001). Δ9-tetrahydrocannabinol increases brain temperature and inverts circadian rhythms. *NeuroReport*, **12,** 3791-3794.

PRICE, L.H., RICAURTE, G.A., KRYSTAL, J.H. & HENINGER, G.R. (1989). Neuroendocrine and mood responses to intravenous L-tryptophan in 3,4-methylenedioxymethamphetamine (MDMA) users. *Archives of General Psychiatry*, **46,** 20-22.

RAMOS, A. & MORMÈDE, P. (1998). Stress and emotionality: a multidimensional and genetic approach. *Neuroscience and Biobehavioral Reviews*, **22,** 33-57.

RAMOS, A., MELLERIN, Y., MORMÈDE, P. & CHAOULOFF, F. (1998). A genetic and multifactorial analysis of anxiety-related behaviours in Lewis and SHR intercrosses. *Behavioural Brain Research*, **96,** 195-205.

RANDALL, T. (1992). Ecstasy-fueled 'rave' parties become dances of death for English youths. *Journal of the American Medical Association*, **268,** 1505-1506.

RANG, H.P., DALE, M.M. & RITTER, J.M. (1995). *Pharmacology*. New York: Churchill Livingstone.

RATTRAY, M. (1991). Ecstasy: towards an understanding of the biochemical basis of the actions of MDMA. *Essays in Biochemistry*, **26,** 77-87.

RENEMAN, L., HABRAKEN, J.B., MAJOIE, C.B., BOOIJ, J. & DEN HEETEN, G.J. (2000a). MDMA ("Ecstasy") and its association with cerebrovascular accidents: preliminary findings. *American Journal of Neuroradiology*, **21,** 1001-1007.

RENEMAN, L., BOOIJ, J., SCHMAND, B., VAN DEN BRINK, W. & GUNNING, B. (2000b). Memory disturbances in "Ecstasy" users are correlated with an altered brain serotonin neurotransmission. *Psychopharmacology*, **148,** 322-324.

REX, A., VOIGT, J.P. & FINK, H. (1999). Behavioral and neurochemical differences between Fischer 344 and Harlan-Wistar rats raised identically. *Behavior Genetics*, **29,** 187-192.

RICAURTE, G., BRYAN, G., STRAUSS, L., SEIDEN, L. & SCHUSTER, C. (1985). Hallucinogenic amphetamine selectively destroys brain serotonin nerve terminals. *Science*, **229,** 986-988.

RICAURTE, G.A., DELANNEY, L.E., WIENER, S.G., IRWIN, I. & LANGSTON, J.W. (1988a). 5-hydroxyindoleacetic acid in cerebrospinal fluid reflects serotonergic damage induced by 3,4-methylenedioxymethamphetamine in CNS of non-human primates. *Brain Research*, **474,** 2359-2363.

RICAURTE, G.A., FORNO, L.S., WILSON, M.A., DELANNEY, L.E., IRWIN, I., MOLLIVER, M.E. & LANGSTON, J.W. (1988b). (±)-3,4-methylenedioxymethamphetamine selectively damages central serotonergic neurons in nonhuman primates. *Journal of the American Medical Association*, **260,** 51-55.

RICAURTE, G.A., DELANNEY, L.E., IRWIN, I. & LANGSTON, J.W. (1988c). Toxic effects of MDMA on central serotonergic neurons in the primate: importance of route and frequency of drug administration. *Brain Research*, **446,** 165-168.

RICAURTE, G.A. & MCCANN, U.D. (1992). Neurotoxic amphetamine analogues: effects in monkeys and implications for humans. *Annals of the New York Academy of Sciences*, **648,** 371-382.

RICAURTE, G.A., MARTELLO, A.L., KATZ, J.L. & MARTELLO, M.B. (1992). Lasting effects of (±)-3,4-methylenedioxymethamphetamine (MDMA) on central serotonergic neurons in nonhuman primates: neurochemical observations. *The Journal of Pharmacology and Experimental Therapeutics*, **261,** 616-622.

RICAURTE, G.A., MCCANN, U.D., SZABO, Z. & SCHEFFEL, U. (2000). Toxicodynamics and long-term toxicity of the recreational drug, 3, 4-methylenedioxymethamphetamine (MDMA, 'Ecstasy'). *Toxicology Letters*, **112-113,** 143-146.

RICHARDSON, R. (2000). Shock sensitization of startle: learned or unlearned fear? *Behavioural Brain Research*, **110,** 109-117.

RODGERS, R.J. *(1997). Animal* models of 'anxiety': where next? *Behavioural Pharmacology*, **8,** 477-496.

RODGERS, R.J. & DALVI, A. (1997). Anxiety, defence and the elevated plus-maze. *Neuroscience and Biobehavioral Reviews*, **21,** 801-810.

ROTH, B.L., WILLINS, D.L., KRISTIANSEN, K. & KROEZE, W.K. (1998). 5-hydroxytryptamine$_2$-family receptors (5-hydroxytryptamine$_{2A}$, 5-hydroxytryptamine$_{2B}$, 5-hydroxytryptamine$_{2C}$): where structure meets function. *Pharmacology and Therapeutics*, **79,** 231-257.

ROTHMAN, R.B., MELE, A., REID, A.A., AKUNNE, H.C., GREIG, N., THURKAUF, A., DE COSTA, B.R., RICE, K.C. & PERT, A. (1991). GBR 12909 antagonizes the ability of cocaine to elevate extracellular levels of dopamine. *Pharmacology Biochemistry and Behavior*, **40,** 387-397.

RUDNICK, G. (1977). Active transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets. *Journal of Biological Chemistry*, **252,** 2170-2174.

RUDNICK, G. & WALL, S.C. (1992). The molecular mechanism of "ecstasy" [3,4-methylenedioxymethamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. *Proceedings of the National Academy of Sciences of the United States of America*, **89,** 1817-1821.

RUDNICK, G. & WALL, S.C. (1993). Non-neurotoxic amphetamine derivatives release serotonin through serotonin transporters. *Molecular Pharmacology*, **43,** 271-276.

RUDNICK, G. (1997). Mechanisms of biogenic amine neurotransmitter transporters. In: *Neurotransmitter transporters. Structure, function and regulation*, pp. 73-100. Editor: Reith, M.E.A. Totowa: Humana Press.

SABOL, K.E., LEW, R., RICHARDS, J.B., VOSMER, G.L. & SEIDEN, L.S. (1996). Methylenedioxymethamphetamine-induced serotonin deficits are followed by partial recovery over a 52-week period. Part I: synaptosomal uptake and tissue concentrations. *The Journal of Pharmacology and Experimental Therapeutics*, **276,** 846-854.

SABOL, K.E. & SEIDEN, L.S. (1998). Reserpine attenuates D-amphetamine and MDMA-induced transmitter release in vivo: a consideration of dose, core temperature and dopamine synthesis. *Brain Research*, **806,** 69-78.

SALMI, P. & AHLENIUS, S. (1998). Evidence for functional interactions between 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in rat thermoregulatory mechanisms. *Pharmacology and Toxicology*, **82,** 122-127.

SÁNCHEZ, C. (1995). Serotonergic mechanisms involved in the exploratory behaviour of mice in a fully automated two-compartment black and white test box. *Pharmacology and Toxicology*, **77,** 71-78.

SANCHEZ, V., CAMARERO, J., ESTEBAN, B., PETER, M.J., GREEN, A.R. & COLADO, M.I. (2001). The mechanisms involved in the long-lasting neuroprotective effect of fluoxetine against MDMA ('ecstasy')-induced degeneration of 5-HT nerve endings in rat brain. *British Journal of Pharmacology*, **134,** 46-57.

SCANZELLO, C.R., HATZIDIMITRIOU, G., MARTELLO, A.L., KATZ, J.L. & RICAURTE, G.A. (1993). Serotonergic recovery after (±)3,4-(methylenedioxy) methamphetamine injury: observations in rats. *The Journal of Pharmacology and Experimental Therapeutics*, **264,** 1484-1491.

SCEARCE-LEVIE, K., VISWANATHAN, S.S. & HEN, R. (1999). Locomotor response to MDMA is attenuated in knockout mice lacking the 5-HT_{1B} receptor. *Psychopharmacology*, **141,** 154-161.

SCHEFFEL, U., LEVER, J.R., STATHIS, M. & RICAURTE, G.A. (1992). Repeated administration of MDMA causes transient down-regulation of serotonin 5-HT_2 receptors. *Neuropharmacology*, **31,** 881-893.

SCHEFFEL, U., SZABO, Z., MATHEWS, W.B., FINLEY, P.A., DANNALS, R.F., RAVERT, H.T., SZABO, K., YUAN, J. & RICAURTE, G.A. (1998). In vivo detection of short- and long-term MDMA neurotoxicity - a positron emission tomography study in the living baboon brain. *Synapse*, **29,** 183-192.

SCHIFANO, F. (1991). Chronic atypical psychosis associated with MDMA ('ecstasy') abuse. *The Lancet*, **338,** 1335.

SCHMIDT, C.J., WU, L. & LOVENBERG, W. (1986). Methylenedioxymethamphetamine: a potentially neurotoxic amphetamine analogue. *European Journal of Pharmacology*, **124,** 175-178.

SCHMIDT, C.J. (1987a). Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine. *The Journal of Pharmacology and Experimental Therapeutics*, **240,** 1-7.

SCHMIDT, C.J. (1987b). Acute administration of methylenedioxymethamphetamine: comparison with the neurochemical effects of its N-desmethyl and N-ethyl analogs. *European Journal of Pharmacology*, **136,** 81-88.

SCHMIDT, C.J., LEVIN, J.A. & LOVENBERG, W. (1987). *In vitro* and *in vivo* neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain. *Biochemical Pharmacology*, **36,** 747-755.

SCHMIDT, C.J. & TAYLOR, V.L. (1988). Direct central effects of acute methylenedioxymethamphetamine on serotonergic neurons. *European Journal of Pharmacology*, **156,** 121-131.

SCHMIDT, C.J. & KEHNE, J.H. (1990). Neurotoxicity of MDMA: neurochemical effects. *Annals of the New York Academy of Sciences*, **600,** 665-681.

SCHMIDT, C.J., BLACK, C.K., ABBATE, G.M. & TAYLOR, V.L. (1990a). Methylenedioxymethamphetamine-induced hyperthermia and neurotoxicity are independently mediated by 5-HT_2 receptors. *Brain Research*, **529,** 85-90.

SCHMIDT, C.J., ABBATE, G.M., BLACK, C.K. & TAYLOR, V.L. (1990b). Selective 5-hydroxytryptamine$_2$ receptor antagonists protect against the neurotoxicity of methylenedioxymethamphetamine in rats. *The Journal of Pharmacology and Experimental Therapeutics*, **255,** 478-483.

SCHMIDT, C.J., BLACK, C.K. & TAYLOR, V.L. (1990c). Antagonism of the neurotoxicity due to a single administration of methylenedioxymethamphetamine. *European Journal of Pharmacology*, **181,** 59-70.

SCHMIDT, C.J., TAYLOR, V.L., ABBATE, G.M. & NIEDUZAK, T.R. (1991). 5-HT_2 antagonists stereoselectively prevent the neurotoxicity of 3,4-methylenedioxymethamphetamine by blocking the acute stimulation of dopamine synthesis: reversal by L-Dopa. *The Journal of Pharmacology and Experimental Therapeutics*, **256,** 230-235.

SCHMIDT, C.J., FADAYEL, G.M., SULLIVAN, C.K. & TAYLOR, V.L. (1992). 5-HT_2 receptors exert a state-dependent regulation of dopaminergic function: studies with MDL 100,907 and the amphetamine analogue, 3,4-methylenedioxymethamphetamine. *European Journal of Pharmacology*, **223,** 65-74.

SCHMITT, U. & HIEMKE, C. (1998a). Strain differences in open-field and elevated plus-maze behavior of rats without and with pretest handling. *Pharmacology Biochemistry and Behavior*, **59,** 807-811.

SCHMITT, U. & HIEMKE, C. (1998b). Combination of open field and elevated plus-maze: a suitable test battery to assess strain as well as treatment differences in rat behavior. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **22,** 1197-1215.

SCHWARTING, R.K.W., THIEL, C.M., MÜLLER, C.P. & HUSTON, J.P. (1998). Relationship between anxiety and serotonin in the ventral striatum. *NeuroReport*, **9,** 1025-1029.

SCREATON, G.R., CAIRNS, H.S., SARNER, M., SINGER, M., THRASHER, A. & COHEN, S.L. (1992). Hyperpyrexia and rhabdomyolysis after MDMA ('ecstasy') abuse. *The Lancet*, **339,** 677-678.

SEMPLE, D.M., EBMEIER, K.P., GLABUS, M.F., O'CARROLL, R.E. & JOHNSTONE, E.C. (1999). Reduced *in vivo* binding to the serotonin transporter in the cerebral cortex of MDMA ('ecstasy') users. *British Journal of Psychiatry*, **175,** 63-69.

SETEM, J., PINHEIRO, A.P., MOTTA, V.A., MORATO, S. & CRUZ, A.P.M. (1999). Ethopharmacological analysis of 5-HT ligands on the rat elevated plus-maze. *Pharmacology Biochemistry and Behavior*, **62,** 515-521.

SHANKARAN, M. & GUDELSKY, G.A. (1998). Effect of 3,4-methylenedioxymethamphetamine (MDMA) on hippocampal dopamine and serotonin. *Pharmacology Biochemistry and Behavior*, **61,** 361-366.

SHANKARAN, M. & GUDELSKY, G.A. (1999). A neurotoxic regimen of MDMA suppresses behavioral, thermal and neurochemical responses to subsequent MDMA administration. *Psychopharmacology*, **147,** 66-72.

SHANKARAN, M., YAMAMOTO, B.K. & GUDELSKY, G.A. (1999a). Involvement of the serotonin transporter in the formation of hydroxyl radicals induced by 3,4-methylenedioxymethamphetamine. *European Journal of Pharmacology*, **385,** 103-110.

SHANKARAN, M., YAMAMOTO, B.K. & GUDELSKY, G.A. (1999b). Mazindol attenuates the 3,4-methylenedioxymethamphetamine-induced formation of hydroxyl radicals and long-term depletion of serotonin in the striatum. *Journal of Neurochemistry*, **72,** 2516-2522.

SHANKARAN, M., YAMAMOTO, B.K. & GUDELSKY, G.A. (2001). Ascorbic acid prevents 3,4-methylenedioxymethamphetamine (MDMA)-induced hydroxyl radical formation and the behavioral and neurochemical consequences of the depletion of brain 5-HT. *Synapse*, **40,** 55-64.

SIMPSON, D.L. & RUMACK, B.H. (1981). Methylenedioxymethamphetamine. Clinical description of overdose, death, and review of pharmacology. *Archives of Internal Medicine*, **141,** 1507-1509.

SLIKKER JR., W., ALI, S.F., SCALLET, A.C., FRITH, C.H., NEWPORT, G.D. & BAILEY, J.R. (1988). Neurochemical and neurohistological alterations in the rat and monkey produced by orally administered methylenedioxymethamphetamine (MDMA). *Toxicology and Applied Pharmacology*, **94,** 448-457.

SLIKKER JR., W., HOLSON, R.R., ALI, S.F., KOLTA, M.G., PAULE, M.G., SCALLET, A.C., MCMILLAN, D.E., BAILEY, J.R., HONG, J.S. & SCALZO, F.M. (1989). Behavioral and neurochemical effects of orally administered MDMA in the rodent and nonhuman primate. *NeuroToxicology*, **10,** 529-542.

SOLOWIJ, N., HALL, W. & LEE, N. (1992). Recreational MDMA use in Sydney: a profile of 'Ecstasy' users and their experiences with the drug. *British Journal of Addiction*, **87,** 1161-1172.

SONSALLA, P.K., NICKLAS, W.J. & HEIKKILA, R.E. (1989). Role for excitatory amino acids in methamphetamine-induced nigrostriatal dopaminergic toxicity. *Science*, **243,** 398-400.

SONSALLA, P.K., RIORDAN, D.E. & HEIKKILA, R.E. (1991). Competitive and non-competitive antagonists at N-methyl-D-aspartate receptors protect against methamphetamine-induced dopaminergic damage in mice. *The Journal of Pharmacology and Experimental Therapeutics*, **256,** 506-512.

SPANOS, L.J. & YAMAMOTO, B.K. (1989). Acute and subchronic effects of methylenedioxymethamphetamine [(±)MDMA] on locomotion and serotonin syndrome behaviour in the rat. *Pharmacology Biochemistry and Behavior*, **32,** 835-840.

STEELE, T.D., MCCANN, U.D. & RICAURTE, G.A. (1994). 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy"): pharmacology and toxicology in animals and humans. *Addiction*, **89,** 539-551.

STONE, D.M., STAHL, D.C., HANSON, G.R. & GIBB, J.W. (1986). The effects of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain. *European Journal of Pharmacology*, **128,** 41-48.

STONE, D.M., HANSON, G.R. & GIBB, J.W. (1987a). Differences in the central serotonergic effects of methylenedioxymethamphetamine (MDMA) in mice and rats. *Neuropharmacology*, **26,** 1657-1661.

STONE, D.M., MERCHANT, K.M., HANSON, G.R. & GIBB, J.W. (1987b). Immediate and long-term effects of 3,4-methylenedioxymethamphetamine on serotonin pathways in brain of rat. *Neuropharmacology*, **26,** 1677-1683.

STONE, D.M., JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1988). Role of endogenous dopamine in the central serotonergic deficits induced by 3,4-methylenedioxymethamphetamine. *The Journal of Pharmacology and Experimental Therapeutics*, **247,** 79-87.

STRANGE, P.G. (2000). Dopamine receptors: www.tocris.com.

SUGRUE, M.F. (1984). Antagonism of fenfluramine-induced hyperthermia in rats by some, but not all, selective inhibitors of 5-hydroxytryptamine uptake. *British Journal of Pharmacology*, **81,** 651-657.

TARASKA, T. & FINNEGAN, K.T. (1997). Nitric oxide and the neurotoxic effects of methamphetamine and 3,4-methylenedioxymethamphetamine. *The Journal of Pharmacology and Experimental Therapeutics*, **280,** 941-947.

TEHAN, B. (1993). Ecstasy and dantrolene. *British Medical Journal*, **306,** 146.

TREIT, D. (1985). Animal models for the study of anti-anxiety agents: a review. *Neuroscience and Biobehavioral Reviews*, **9,** 203-222.

TREIT, D., MENARD, J. & ROYAN, C. (1993). Anxiogenic stimuli in the elevated plus-maze. *Pharmacology Biochemistry and Behavior*, **44,** 463-469.

TUCKER, G.T., LENNARD, M.S., ELLIS, S.W., WOODS, H.F., CHO, A.K., LIN, L.Y., HIRATSUKA, A., SCHMITZ, D.A. & CHU, T.Y.Y. (1994). The demethylenation of methylenedioxymethamphetamine ("ecstasy") by debrisoquine hydroxylase (CYP2D6). *Biochemical Pharmacology*, **47,** 1151-1156.

TYE, N.C., EVERITT, B.J. & IVERSEN, S.D. (1977). 5-hydroxytryptamine and punishment. *Nature*, **268,** 741-743.

TYE, N.C., IVERSEN, S.D. & GREEN, A.R. (1979). The effects of benzodiazepines and serotonergic manipulations on punished responding. *Neuropharmacology*, **18,** 689-695.

UPHOUSE, L. (1997). Multiple serotonin receptors: too many, not enough, or just the right number? *Neuroscience and Biobehavioral Reviews*, **21,** 679-698.

VOGEL, J.R., BEER, B. & CLODY, D.E. (1971). A simple and reliable conflict procedure for testing anti-anxiety agents. *Psychopharmacologia*, **21,** 1-7.

WALLACE, T.L., GUDELSKY, G.A. & VORHEES, C.V. (2001). Alterations in diurnal and nocturnal locomotor activity in rats treated with a monoamine-depleting regimen of methamphetamine; or 3,4-methylenedioxymethamphetamine. *Psychopharmacology*, **153,** 321-326.

WEISS, S.M., WADSWORTH, G., FLETCHER, A. & DOURISH, C.T. (1998). Utility of ethological analysis to overcome locomotor confounds in elevated maze models of anxiety. *Neuroscience and Biobehavioral Reviews*, **23,** 265-271.

WHIMBEY, A.E. & DENENBERG, V.H. (1967). Two independent behavioral dimensions in open-field performance. *Journal of Comparative and Physiological Psychology*, **63,** 500-504.

WHITAKER-AZMITIA, P.M. & ARONSON, T.A. (1989). "Ecstasy" (MDMA)-induced panic. *American Journal of Psychiatry*, **146,** 119.

WIESINGER, H. (2001). Arginine metabolism and the synthesis of nitric oxide in the nervous system. *Progress in Neurobiology*, **64,** 365-391.

WILSON, M.A., RICAURTE, G.A. & MOLLIVER, M.E. (1989). Distinct morphologic classes of serotonergic axons in primates exhibit differential vulnerability to the psychotropic drug 3,4-methylenedioxymethamphetamine. *Neuroscience*, **28,** 121-137.

WRIGHT, I.K., HEATON, M., UPTON, N. & MARSDEN, C.A. (1992). Comparison of acute and chronic treatment of various serotonergic agents with those of diazepam and idazoxan in the rat elevated X-maze. *Psychopharmacology*, **107,** 405-414.

YAMAMOTO, B.K. & SPANOS, L.J. (1988). The acute effects of methylenedioxymethamphetamine on dopamine release in the awake-behaving rat. *European Journal of Pharmacology*, **148,** 195-203.

YAMAMOTO, B.K., NASH, J.F. & GUDELSKY, G.A. (1995). Modulation of methylenedioxymethamphetamine-induced striatal dopamine release by the interaction between serotonin and γ-aminobutyric acid in the substantia nigra. *The Journal of Pharmacology and Experimental Therapeutics*, **273,** 1063-1070.

YEH, S.Y. (1999). N-tert-butyl-alpha-phenylnitrone protects against 3,4-methylenedioxymethamphetamine-induced depletion of serotonin in rats. *Synapse*, **31,** 169-177.

ZHANG, Y., MARCILLAT, O., GIULIVI, C., ERNSTER, L. & DAVIES, K.J.A. (1990). The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *The Journal of Biological Chemistry*, **265,** 16330-16336.

ZHAO, Z., CASTAGNOLI JR., N., RICAURTE, G.A., STEELE, T. & MARTELLO, M. (1992). Synthesis and neurotoxicological evaluation of putative metabolites of the serotonergic neurotoxin 2-(methylamino)-1-[3,4-(methylenedioxy)phenyl]propane [(methylenedioxy)methamphetamine]. *Chemical Research in Toxicology*, **5,** 89-94.

ZHENG, Y. & LAVERTY, R. (1998). Role of brain nitric oxide in (±)3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity in rats. *Brain Research*, **795,** 257-263.

ZIMMERMANN, A., STAUFFACHER, M., LANGHANS, W. & WÜRBEL, H. (2001). Enrichment-dependent differences in novelty exploration in rats can be explained by habituation. *Behavioural Brain Research*, **121,** 11-20.

http://dancesafe.org "DanceSafe has two fundamental operating principles: harm reduction and popular education...successful, peer-based educational programs to reduce drug abuse and empower young people to make healthy, informed lifestyle choices."

http://www.drugscope.org.uk "DrugScope is designated by Government as the United Kingdom focal point for drugs information...one element of a European network of drugs information centres."

http://www.erowid.org "Erowid.org is an online library of information about psychoactive plants and chemicals and related topics."

http://www.thesite.org "TheSite is produced and managed by YouthNet UK... TheSite.org aims to offer the best guide to life for young adults, aged 16-25."

http://www.usdoj.gov/dea "The mission of the Drug Enforcement Administration (DEA) is to enforce the controlled substances laws and regulations of the United States."